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THE FUNCTIONAL DEVELOPMENT OF SOME MAMMALIAN NEUROMUSCULAR MECHANISMS

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> "Herophilus leaveth to unborne babes a mooving naturall, but not a respiration, of which motion the sinewes be the instrumentall cause, but afterwards they become perfect living animall creatures, when being come forth of the wombe, they take in breath from the aire."
>
> (Plutarch, from Needham, 1934, p 44.)

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I. INTRODUCTION

THERE are at present at least two divergent views as to how the prenatal behaviour of any mammal develops. According to one view the behaviour of a mammalian embryo begins as a generalized sort of activity, i.e. the reaction of the embryo is much the same to any adequate stimulus, and usually involves the greater portion of the muscular system. Later the response becomes restricted and related to the stimulus so that the reaction of the muscular system becomes for certain stimuli so stereotyped and automatic as to be termed a "reflex". According to a second view the first responses of the embryo are stereotyped and automatic. Only by the gradual combination of all these unit reflexes are the final behaviour patterns established. A simple example may serve to illustrate the different views. Let a 12 in. square made up of one hundred and forty-four individual squares represent the behaviour of an animal. One school would view the large square as having been broken up into the smaller squares, the reflexes. The second school would regard the large square as having been constructed by the aggregation of the many small ones.

The first of these two views when extended into antenatal development resembles very closely the doctrine of the *Gestalt* school of psychology, i.e. that each stimulus is perceived as a whole and in relation to the needs of the organism. Learning is held to be an analytical process proceeding from the whole to the particulate. The second view is allied to the behaviourist theories, i.e. that learning is a synthetic process in which new responses are added to a primary set already present at birth.

So far as the urodele amphibia are concerned the basic researches of Coghill (1929) have demonstrated that the development of behaviour in these forms is in accordance with the first view. Coghill found in Amblystoma that the nervous system develops structurally and functionally in a predictable manner. The behaviour of the larva develops from the beginning through the progressive expansion of a total pattern, while within this total pattern partial patterns acquired varying degrees of independence. Judged, therefore, solely on the evidence derived from this animal the reflex may be regarded as secondary, having been carved out of the total response. Recently these generalizations have been extended to include the development of the activity of Anura (Youngstrom, 1938).

In 1929 Coghill collected some of the literature then available on the activity displayed during prenatal development by mammalian embryos and foetuses, and in his analysis of these observations suggested that the generalizations he had made with regard to the development of behaviour in Amblystoma might also be applicable to the development of behaviour in mammalian forms. This suggestion did not go long unchallenged, but it has served as the impetus for a score of further researches on the activity of mammalian embryos and foetuses in a variety of forms from marsupials to man.

As a result of these researches, inspired by Coghill's studies, a considerable body of information has been accumulated with regard to the development of mammalian neuromuscular mechanisms. The object of the present article is to review some of the facts so brought out, together with some that we have added, not in the light of the behaviour problem which has been kept in the background, but in that of a problem in morphology and physiology, seeking to appreciate those manifestations which the mammals have in common and to enquire into their causal background.

II. THE INACTIVE STAGE OF DEVELOPMENT

(1) *The muscular system*

Aside from the movements incidental to the beat of the heart, all mammalian embryos so far studied are motionless during the very early part of their development. The activity of the mother appears to replace the ciliary activity of the amphibian embryo and the contractions of the smooth muscle of the amniotic sac in birds as mechanisms for agitating the embryo. The length of this inactive period bears little relationship to the total gestation period, for the rat born on the twenty-first or second day is inactive until the sixteenth (Angulo, 1932); the sheep born on about the hundred and fiftieth day remains inactive only until the thirty-fourth day (Barcroft *et al.* 1936).

Toward the close of this inactive period of development, the muscles of the embryo become excitable first to faradic and then later to galvanic currents applied to the skin directly over them when the embryo has been removed from the amniotic sac (see Table I). Until the recent observations of Straus (1938), suggesting that a striated muscle can be made to contract by stimulation of its motor nerve before it contracted to direct electrical stimulation (see also Straus & Weddell, 1938), it was generally accepted that the capacity to respond to electrical stimuli is attained by a muscle fibre before it could be aroused by stimulation of its motor nerve. The effector tissue was thought to be the first link in the chain of the behaviour mechanism to become functional in either the somatic or visceral systems, for in the kitten the dilator muscle of the pupil contracts to direct faradization before it responds to impulses arriving by the post-ganglionic fibres of the superior cervical ganglion (Barron, unpublished). Until the results of Straus appear in detail final judgement must be reserved in the case of somatic muscle.

Table I. *First appearance of direct excitability of muscle*

Animal	Age or size	Type of stimulation	Observers
Chick	60–65 hr.	Faradic currents	Olivio (1925)
Rat	11·0–11·5 mm.	,,	Windle *et al.* (1935)
Cat	11 mm.	,,	Windle *et al.* (1933)
Sheep	32 days	,,	Barcroft *et al.* (1936)

The first muscle fibres of the sheep to reach the stage of electrical excitability in development are situated in the neck region and appear to stretch from the vertebral column to the shoulder girdle, for the first muscle contractions that can be induced by electrical stimulation abduct the whole fore limb (Windle *et al.* 1933). In the cat embryo according to Windle the next muscle fibres to become electrically excitable are also in the neck and in contracting move the head backward and toward the side. The dorsal longitudinal muscles of the back and tail are next in order, followed by muscle fibres that abduct the hind leg as a whole, presumably the future gluteal group. The muscles of the fore limb precede those of the hind limb, and muscle fibres on the proximal segments those of the distal. Functionally, therefore, the muscles of the trunk and limbs appear to develop in general in a cephalocaudal direction and proximodistally. This order is not true of the muscles of the head. In the sheep at least, the muscles of mastication are functionally in advance of those of the tongue and pharynx, but the eye muscles precede the facial muscles in functional development (Barcroft & Barron, 1939). This precocity of the eye muscles may be associated in some way with their supposed origin from head myotomes. The muscles of the head differentiate in another order and at a later date than those of the trunk in the sheep.

Since the histology of muscle fibres correlates so closely with their functional capacity—as Weiss (1899) has demonstrated in Amphibia—the order in which these muscle fibres react to faradic currents might be used as an index of their order of differentiation. Assuming that the same correlation exists in mammals, differentiation of muscle would appear, therefore, to procede cephalocaudally and proximodistally on the trunk, though no generalization appears possible for the head. Variations from this general pattern of the trunk and limb muscles appear, however, in the course of development. In a five-months-old human foetus, for example, Babinski & Onanoff (1888) found the brachioradialis to be the muscle most advanced in its histological development in the forearm, though in general the proximal muscles were more advanced than the distal. On the head the masseter was behind the orbicularis oculi in its differentiation.

The order of the differentiation of the individual muscles has an importance beyond its expression in a physiological response, for muscle can no longer be regarded as playing a passive role in the development of nervous mechanisms. Hamburger (1934) has demonstrated in the chick that both the sensory and motor elements of the spinal cord are influenced by the peripheral field (see also Barron & Barcroft, 1938). Tello (1917) has demonstrated that the appearance of the myoneural junction is dependent upon the state of differentiation of the muscle. But more important still to a study of the development of behaviour mechanisms are the demonstrations by Weiss (1936) that individual muscles are able to "modulate" the anterior horn cells that supply them, and hence to determine the peripheral response. How such influences emanating from the muscular system are impressed upon the central and peripheral nervous systems has yet to be determined, but these observations do emphasize the role of the effector tissue in the development of behaviour. All later phases of the somatic activity of the foetus have, of course, their ultimate and final expression in the contraction of these developing muscles, and so far the body movement produced by this muscular contraction is the only means by which the activity of the central nervous system may be followed, for no direct records of nerve impulses appear to have been made from the motor nerves of embryos and foetuses. For this reason it is important that the physiological properties of the muscle be quite clearly established in order that purely muscular phenomena may be distinguished from neuromuscular.

The first contractions of mammalian skeletal muscles occur in response not to galvanic currents as might have been expected but to faradization. The responses to galvanic currents are first seen some time later (Straus, verbal communication and Barcroft & Barron, unpublished). These first contractions to faradic currents are local and are confined to a region near the electrodes. The latent period has not been recorded for embryonic mammalian muscle, but for the chick muscle it is reported to be of the order of 1 or 2 sec. when it first responds to electrical stimuli, and it has been suggested that this latent period increases with growth (Olivio, 1925). The immature character of foetal mammalian muscle is illustrated by

Krasnogorski's (1914) observation that the undeveloped striated muscle of a premature baby had a latent period of $31{\cdot}6\sigma$. (The latent period of an isolated frog muscle as usually recorded is about 6σ.) The primitive character is also emphasized by Soltmann's (1877) observation that sixteen stimuli per second were adequate to cause a perfect tetanus in muscles of new born rabbits. The contractions are quickly followed by relaxation as there is no tendency for the muscle fibres, in the sheep at least, to remain shortened. This remains true as development advances, so that sustained or tetanic contractions may be assumed to indicate that the anterior horn cells are firing off a series of impulses.

The studies of Minkowski (1928) on the muscles of human embryos and foetuses from the third month onward have demonstrated that foetal muscles may be excited mechanically by tapping them. Shortening in the muscle tapped is often followed by contraction of a neighbouring muscle even after the nerves have been removed and the central connexions are cut. How the excitatory wave spreads from one muscle to another is not clear, but it must be remembered that these muscle fibres may be devoid of sarcolemma in the adult sense (Hewer, 1927). Minkowski has further demonstrated that stretching a foetal striped muscle may act as a stimulus for the contraction of the fibres even in the absence of the spinal cord. As development proceeds these responses to mechanical stimuli become restricted to the muscle primarily affected. Until this stage of differentiation has been attained by muscles, however, it would appear that a muscle contracting in response to motor nerve impulses could set up a chain of non-nervous reactions in neighbouring muscle (see also Wintrebert, 1920; Schaper, 1898). Coordinate activity, however well differentiated the nervous system might be, would appear to be impossible with incompletely developed foetal muscles. The role of the effector tissues in the development of behaviour certainly merits more attention than it has so far received. One of the most lucrative problems in foetal physiology would be an investigation of the nerve muscle complex.

(2) *The nervous system*

During this primary inactive period of development the central and peripheral nervous systems are growing and differentiating in "anticipation" (Barcroft, 1938) of future needs. By the end of this period some definite though miniature fibre bundles have been laid down in the central nervous system. Upon the nature and connexions of these pathways and fibre tracts depends the character not only of the earliest activity of the embryo, but the subsequent activity of the animal after it has been born. Considerable interest therefore attaches itself to the factors that determine this early differentiation of the nervous system. A consideration of these factors lies outside the scope of this review and an excellent discussion is available by Detwiler (1936).

The structural development of the nervous system has been described, in a number of forms, by Windle and his co-workers (see Windle, 1931, 1934; Windle & Baxter, 1936; Windle & Fitzgerald, 1937; Scharpenberg & Windle, 1938) and by Bok (1915). Accounts have also appeared by Angulo (1936*a*, 1939) on the rat, and

Pankratz (1936) on the rabbit. Though the extent to which the entire nervous system has developed by the end of the non-motile stage undoubtedly varies from one animal form to another, the structure of the medulla and the spinal cord does appear to possess some features common to all forms.

At the end of the inactive period of development motor and sensory fibres are distributed from the spinal cord and medulla to peripheral structures. Dorsal, lateral and ventral funiculi and an anterior commissure are present at all levels of the spinal cord. Collaterals from the dorsal funiculus penetrate into the alar lamina, but in none of the forms so far described do they appear to establish direct connexions with the anterior horn cells of the same side. The anterior commissure in the pre-motile stage is composed of fibres from several sources. Dendrites are contributed by the medial motor cell column. The axones arise from association cells. Some of these cells are located in the anterior horn, others lie lateral to the central canal and a third group is situated in the future dorsal cell columns. The majority of these axones form a contralateral ascending tract of short association neurones, linking segments together (Windle, 1933). According to Angulo (1936*a*) the ventral or anterior commissure contains still another group of fibres, i.e. collaterals of the axones of a descending system of fibres, the ventral longitudinal bundle.

The fibre elements that compose the ventral funiculus are still in dispute; according to one view they are almost wholly ascending association elements, to another they contain the ascending association and in addition descending association or internuncial fibres. The cell bodies of some of these descending association elements are in the medulla (Angulo, 1939) and may contribute to the formation of the future medial longitudinal fasciculus as well as the reticulo-spinal system. Shaner (1932) illustrates the medial longitudinal fasciculus as continuous with the ventral fibre columns of the spinal cord in the 20 mm. pig. Angulo (1936*a*, 1939) has described this tract or fibre bundle as present in the premotile rat. Certain it is that, in the chick, it makes its appearance very early, and acts as a pathway of coordination prior to the advent of a sensory system (Windle & Orr, 1934). Though its appearance has not been established finally in premotile mammalian forms some such system is certainly functioning as a caudal conducting system very early in ontogeny, indeed, and it is convenient to assume even in the absence of final positive evidence that this system is the caudal extension of the medial longitudinal fasciculus.

In summary, therefore, at the end of the inactive stage of development, the embryo is in possession of muscular tissue, at least some of which has attained electrical excitability. Into these muscles are distributed sensory and motor fibres. The anterior horn cells in the spinal cord are related to two sets of internuncial neurones, one descending from the region of the hind brain via the caudal extension of the medial longitudinal fasciculus and a second arising segmentally and running intersegmentally. These internuncial systems are in turn in anatomical relation to some of the afferent fibres of the head and the trunk respectively. The individual sensory trunks discharging into each system will be discussed later. Without any perceptible alteration in structural features, some of these elements combine to convert the embryo from an inactive aggregate of tissues into an organism, from an *inactive* to an *active* embryo.

III. THE ACTIVE STAGE OF DEVELOPMENT

(1) *"Spontaneous" activity as seen in mammalian embryos*

The first active movements of the mammalian embryo appear shortly after some of its muscles have become electrically excitable. These movements occur in all forms—though to a limited degree in the rat (Windle *et al.* 1935)—and are best seen if the uterus is quickly incised and the embryo exposed in the intact amniotic sac. Difficult to observe and to describe, they last but a few seconds in young embryos and are usually termed "spontaneous", since the causal factor in their production is so far undetermined. In older embryos and foetuses these movements persist for longer and longer periods of time and finally blend with activity which is certainly induced by stimulation of sensory nerves. In older foetuses that present both "spontaneous" and "induced" activity the latter always outlasts the former and reflexes can be obtained after the "spontaneous" activity has ceased. The rat may be an exception to this rule.

When one considers the technical difficulties under which early mammalian embryos are observed the accounts of the "spontaneous" activity are remarkable for their similarity, and it seems not improbable that some of the differences that do appear are to be assigned to experimental conditions. The earliest "spontaneous" movement (see Windle & Griffin, 1931; Carmichael, 1934; Preyer, 1885; Barcroft & Barron, 1939) is usually a slow ventrolateral bending of the head and thoracic trunk so that the head is moved laterally and at the same time rotated (see Table II).

Table II. *First appearance of "spontaneous" activity*

Animal	Age or size	Movement	Observers
Chick	4½ days	Ventro-flexion of trunk	Windle & Orr (1934)
Carrier pigeon	105 ± 5 hr.	Extension and flexion of head	Tuge (1937)
Cat	24 + days	Ventrolateral head flexion	Windle & Griffin (1931)
Sheep	35 days 23 mm.	Head flexed laterally and foreleg abducted	Barcroft & Barron (unpublished)
Guinea pig	28 days	Lateral bending of head and flexion of foreleg	Carmichael (1934)

This movement is in some forms—guinea pig and sheep—associated with movements of the foreleg from the first. In other forms this foreleg movement appears definitely later. As development of the embryo proceeds, the movements of the head, trunk and forelimb become more extensive and the differentiation of the limb allows for flexion and extension of the elbow, and later similar movements occur at the wrist. Movements of the trunk and hind limbs appear next in that order. The "spontaneous" activity spreads cephalocaudally and proximodistally in much the same fashion as the differentiation of the muscles. In the sheep embryo the diaphragm contracts as a part of this early generalized activity, but this appears to be a special feature since diaphragmatic movements have not yet been described even as part of the induced activity of some forms, notably the rat (Straus, verbal communication). The muscles moving the lower jaw are next incorporated as a part of the spontaneous activity and the mouth opens when the activity is at its height. The movements of the infra-hyoid muscles and the tongue follow those of the lower jaw and precede movements of the eyes. The facial musculature, however, appears to take no part in the spontaneous movement.

To the very first movements of the embryo it would be difficult, indeed, to ascribe a purpose, but as more and more muscles are incorporated it is evident that these "spontaneous" movements are directed in at least two ways: toward getting up or "righting" and in "respiratory" movements—obviously no air is taken into the lungs (see Carmichael, 1934). The righting movements have been described for the cat by Windle & Griffin (1931). Similar movements, perhaps even more definitely directed towards getting up, have been seen in the sheep embryo in which, during the early part of development at least, they are closely linked with respiratory movements (Barcroft & Barron, 1936).

The sheep embryo, soon after it reaches the age at which spontaneous movements are first observed, may be seen to flex the trunk laterally. At the same time the head is extended, the forelegs are abducted and the hind legs are extended. The attitude assumed by the embryo, as a result of this movement, is not maintained; the moving parts return quickly to their original resting position so that the movement may be repeated several times, in a rhythmic fashion. With each beat of the rhythm the liver is pushed backward by the diaphragm and in older embryos the intercostal muscles contract. As development advances the effort resembles more and more the gasp of an animal poisoned by minimal lethal doses of hydrocyanic acid vapour (Barcroft, 1934).

At first the movements of the diaphragm are definitely linked with the general extensor activity, but this connexion is soon lost. The rhythmic contractions of the diaphragm often outlast the rhythmic extensor response and are accompanied instead by flexion of the neck, contraction of the infra-hyoid muscles and opening of the mouth. These rhythmic contractions might be considered to be in the nature of an after-discharge, for their duration, their strength and their frequency is intimately related to the amount of the general activity.

Contracting first, therefore, as a part of a general mass movement the muscles concerned in respiration gradually acquire a partial independence, but they always remain linked to the general activity of the embryo or foetus in a manner similar to the conditions existing in the adult. In quiet "respiratory" activity the linkage is not apparent for only the diaphragm contracts. However, as the respiratory efforts become laboured more and more muscles are involved. First the intercostals, next the infra-hyoid muscles and then those opening the mouth come into play in that order. Finally the extensor movement originally associated with the contraction of the diaphragm occurs as a part of what appears to be an inspiratory effort of the foetus. As the respiratory activity again diminishes the muscles drop out in the reverse order. Were we seeking to illustrate Coghill's thesis that partial patterns of activity develop within a total pattern the activity of the diaphragm would serve as an excellent example.

These "spontaneous" respiratory movements have not been as frequently observed as those concerned in getting up, for as stated above the "spontaneous activity" may also be directed toward righting the embryo or foetus. The first undulatory movements of the head are soon accompanied by movements of the limbs. If the snout is rotated toward the right, the right forelimb may be abducted and extended. In older foetuses it is possible to determine that the opposite forelimb is flexed. These attempts to get up are never completely successful, but they do demonstrate that the position of the head of the foetus may determine the attitude of the limbs, for if the head is extended the forelimbs are extended, if the head be flexed the forelegs are flexed. In the early stages of development the movements of the limbs appear for the most part to be firmly linked to the movements of the neck and head. Like the diaphragm, however, they acquire an increasing amount of autonomy in later development.

Both of these behaviour patterns—respiration and getting up—appear first in development as a part of the "spontaneous" activity. In later development, however, it is obvious that the stimuli which arouse the righting efforts arise in the periphery and reach the central nervous system via primary afferent neurones. Particular interest is attached, however, to the causal factors of the early movements of respiration. Are these early respiratory movements first aroused by afferent impulses or are they the first expression of an autonomic mechanism which persists as the "respiratory centre" in the adult?

(2) *Probable mechanisms responsible for "spontaneous" activity*

There appear to be at least three possible ways in which the early "spontaneous" movements, including those of the respiratory muscles, might be produced: (1) by the contraction of muscle fibres in the absence of stimuli via motor nerves (*myogenic*); (2) by stimulation of nerve cells directly through substances in the blood (*neurogenic*); and finally (3) by stimulation of sensory nerves (*reflexogenic*) in which case these movements would not differ from the induced activity of the embryo.

In the lower vertebrate forms the first spontaneous movements are definitely myogenic, for they occur before the motor nerves have grown into the myotomes. In the selachians these rhythmic movements begin in the head region first and later spread backwards along the body to include the whole trunk. When the motor nerves do finally grow into the myotomes the original activity is suppressed. If, however, the nervous system is removed, the rhythmic contractions of the muscles begin again, after an initial period of shock (Wintrebert, 1920). A similar capacity to contract rhythmically in the absence of nervous influences is apparently possessed by amphibian muscle at least before metamorphosis, for Schaper (1898) has described swimming movements in tadpoles in which the nervous system had failed to develop as a result of operative interference. In birds there is apparently no direct evidence to indicate a myogenic stage of activity in ontogeny. Tuge, however, has demonstrated that certain of these "spontaneous movements" of the chick do persist after curare has been injected into the circulation. These movements Tuge (1937) has assumed to be produced by the activity of muscles isolated from nervous influences. Similar experiments involving the use of curare were carried out by Angulo (1935), and though muscles were shown to be directly excitable, no evidence of myogenic activity was presented. In fact, even the observations of Tuge indicating a myogenic stage of activity in the chick embryo must be taken with some reservation, for the action of curare on the immature myoneural junction is not definitely known. Preyer (1885) pointed out that in young embryos very large doses of curare were required to be effective. Further, since these earliest movements occur at a time when the motor nerve endings are still epilemmal (Tello, 1917) as they are in unstriped muscle, the effects of curare might be expected to resemble those on adult unstriped muscle rather than on striped.

Spontaneous contractions of mammalian skeletal muscle fibres grown in tissue culture quite unlike those seen in either heart muscle or the muscle of the chick amnion have been described by Lewis (1920). That such contractions occur in the actual embryo, however, has not been established. But they may be suppressed or inhibited by the presence of nerve fibres after the manner by which the rhythmic myogenic movements of fish embryos are suppressed when motor nerve fibres enter the myotome. Though a true myogenic phase of activity has been found in fishes, amphibia, and described in birds, the precocious development of the nervous system may mask its appearance in mammalian forms. If this supposition is correct, myogenic activity might be expected to follow the experimental removal of the influence of the nervous system on embryonic muscle.

In this connexion Windle *et al.* (1934, 1935) have observed that slow rhythmic contractions do occur in the muscles of very young cat embryos after the heart has stopped beating. They are best seen when the embryo has been cooled and bled. Under these conditions Windle (1939) has shown, as Mines had thirty years earlier (1908) for amphibian muscle, that embryonic muscles can be made to contract spontaneously by changing the calcium and potassium concentrations in the saline solutions bathing them. These rhythmic contractions may be the mammalian homologue of the myogenic activity in fishes.

The second possibility considered above was one in which these spontaneous movements were due to the contraction of skeletal muscle in response to impulses arriving via their motor nerves. In these circumstances the motor horn cells are assumed to be stimulated by changes in the concentration of some of the ions in the fluid media by which they are bathed, and not by effects—chemical or electrical—transmitted to the central terminations of nerve fibres. This idea that alterations in the concentration of constituents of the fluid media bathing the cells of the central nervous system can actually stimulate them is a part of current thought with regard to the initiation and control of the respiratory movements in mammals.

In keeping with this suggestion motor neurones are quite probably genetically related to neurosensory cells—cells that are not only receptor but may also be directly connected with body musculature (Kappers *et al.* 1936). Moreover, the physiological properties of motor neurones have been shown to be remarkably similar to those of sensory endings (Barron & Matthews, 1938). However, Graham Brown (1915) was perhaps the first to offer experimental evidence that the activity of the cells of the spinal cord as well as those of the respiratory centre could be increased by the accumulation of metabolites in the blood (blood stimulus). Graham Brown observed that foetal kittens near full term, when delivered into a bath of physiological saline with both the sides of the placental circulation intact, executed rhythmic movements of progression and respiration when the foetuses were asphyxiated by interruption of the umbilical circulation. The inference drawn from these experiments was that a specific rhythmic activity of the central nervous system could be evoked by asphyxiation before it could be aroused or conditioned by any rhythmic self-generated peripheral stimuli. Results very similar were presented by Angulo (1930), who found that if the circulation through the umbilical vessels of a rat foetus was arrested the foetus would recapitulate the maximum degree of physiological activity it had achieved, but in the reverse order to that in which it had been acquired. For example, foetuses of about 18 days that could not be aroused by tactile stimulation of the hand while the circulation was intact responded spontaneously with waving of the hand shortly after ligation of the umbilical vessels. These discrete movements were followed by mass movements. From these and other similar experiments Angulo (1933) formulated the following law: "Endogenous stimulations affect directly the motor centres and act first on the most recently developed units". This order of response Angulo suggested was due to the fact that the new growing mechanisms have the highest physiologic gradient and are therefore first affected by the relative increase in the metabolites or any stimulating substance in the blood. As one of these stimulating agents that might accumulate Angulo suggested carbon dioxide.

This same suggestion that carbon dioxide if allowed to accumulate might stimulate motor neurones directly had been made earlier by Tracy (1926). In his studies on the development of the toad fish, Tracy found that before the motor system, composed of motor neurones and muscles, had been captured by the sensory system, the activity of the toad fish could be increased by alterations in the fluid environment of the embryo. Tracy believed, though he did not prove, that this increased activity was due to the excitation of the motor system by internal non-nervous agents. One of these agents of preneural stimulation was assumed to be accumulated carbon dioxide (see also White, 1915).

These observations are the basis for the suggestion that motor nerve cells can be directly excited by non-nervous activities. As stated, earlier movements thought to have been produced in this way have been termed "neurogenic" and Coghill (1931) has presented the viewpoint, chiefly upon the basis of Tracy's work, that there is ground for regarding the neurogenic activity of an organism as a part of its overt behaviour in addition to the usual reflexogenic activity produced by the stimulation of sensory nerves.

However, final proof is still wanting that carbon dioxide or indeed any metabolite accumulated during asphyxiation can stimulate a motor cell directly, for as yet no quantitative data have been offered. Asphyxiation of a foetus as produced by ligature of the umbilical vessels must be a combination of oxygen want and carbon dioxide excess. Hence the increased activity might be due to the former as well as the latter, for oxygen want produces gasping in animals, but the accumulation of carbon dioxide never has this basic effect. But even in this instance the possibility exists that the oxygen want impaired the function of certain motor elements rather than exciting others. In this connexion the following observations are of interest.

In the sheep (Barcroft & Barron, 1936, 1937*b*) occlusion of the umbilical circulation does not arouse nor increase the activity of the embryo until about one-third of the gestation period has passed. During part of this time, however, the foetus exhibits spontaneous movements and in the latter part of the period normal reflex activity. There is no evidence during this early period to indicate that asphyxiation has any effect upon the foetus except to hasten its death. During the second third of the gestation period, however, the activity of the foetus, both spontaneous and induced, demonstrated during the first third becomes suppressed or inhibited. That portion of the activity that has been suppressed—no more—can be released or made to reappear if the foetus is asphyxiated. Since this activity when present earlier was not initiated by asphyxiation, it seems probable that its reappearance is produced by the asphyxiation of the neural elements responsible for the suppression or inhibition. This supposition receives support from the fact that a similar release of the suppresssed activity can be brought about by surgical removal of parts of the brain stem (Barcroft & Barron, 1937*b*). Hence it appears probable that in none of the cases described above did the accumulation of metabolites activate neural elements but inactivated rather units that were responsible for inhibition.

This same explanation—that accumulated metabolites render neurones non-functional rather than exciting motor ones—might be extended to the observations of Tracy (1926) and White (1915). The argument would run as follows: Wintrebert has shown that the arrival of motor fibres in the myotome suppresses the spontaneous myogenic activity seen in fish embryos. If the nervous influence is removed surgically, the myogenic activity reappears. Since carbon dioxide may be considered as having a paralysing effect upon nerve cells, the increase in the carbon dioxide in the experiments of White and Tracy may have removed the influence of the motor cells upon the myotome and allowed the myogenic activity to reappear. Until such an explanation has been ruled out and direct quantitative evidence for the stimulating effect of metabolites is advanced, the spontaneous movements seen in early mammalian embryos cannot be accepted as being "neurogenic", nor does it appear necessary to postulate "neurogenic" movements as a positive aspect of behaviour equivalent to reflexogenic activity.

The third and most probable explanation of the "spontaneous" movements is that they are due to the activation of the central nervous and the muscular systems in the adult manner, i.e. by the arrival in the central nervous system of impulses via sensory nerves. They would therefore be allied from the beginning to the activity induced by stimulating sensory nerves. The "spontaneous" movements are best seen immediately after the delivery of the embryo from the uterus, while it is still in the intact amnion. Delivery unavoidably subjects the embryo to pressure changes and to changes of position. This manipulation appears to be responsible for the activity, for transillumination of the pregnant uterus of the sheep demonstrates that so long as the uterus is unmolested the embryo is inactive. In most forms as well, the "spontaneous activity" makes its appearance just shortly before or at the same time in the life of the embryo as induced activity. Since these movements pass over imperceptibly into those induced by local stimuli, it seems probable that the manipulation of delivery of the amniotic sac serves to stimulate a great proportion of the sensory fibres of the embryo. The concerted activity of these fibres may serve to arouse central neurones that would not be excited by the smaller number of fibres affected by a localized stimulus. However, as the ease with which the motor neurones become depolarized increases (Sjöstrand, 1939), a weaker stimulus would be adequate to activate the same motor horn cells, and localized responses could be elicited.

This brief survey of the evidence demonstrates that no final statement can be made as to the nature of the very earliest embryonic activity. The evidence has been sufficient, however, to indicate that the first rhythmic respiratory movements are first activated not by blood-borne stimuli but by afferent impulses.

IV. THE REACTIVE STAGE OF DEVELOPMENT

(1) *The first embryonic reflexes*

The growth and differentiation of the muscular and nervous systems have not progressed far beyond the stage in development at which the "spontaneous" movements first occurred before the application of discrete and localized stimuli—mechanical or electrical—is followed by activity of the embryo. This activity appears to be reflex in nature, that is, it depends for its occurrence upon the activity of at least muscle tissue, motor and sensory neurones. The immediate transition from an unresponsive to a responsive stage in ontogeny is probably due to some physiological change in the structural elements already present, for East (1931) was unable to find any definite anatomical changes associated with the change from the active to the reactive state. The nature of this physiological change is so far undetermined, but it might be simply an increase in the excitability of some one of the elements concerned in the reflex. Pankratz (1931) has suggested that the onset of the activity of the adrenal gland and the consequent release of adrenalin into the circulation might be responsible for a general increase in the excitability of the embryonic muscle tissues at this time.

The first muscles to take part in these reflexes are located in the cervical region. They appear to stretch between the skull, the upper cervical vertebrae, and the shoulder girdle, for these first movements involve either the head, or the foreleg or both. These muscles—the first in the embryo to become electrically excitable—are represented in the adult by the trapezius, the levator scapulae and the rhomboids. The motor neurones involved in the first reflexes have not been determined, but they must have their cell bodies in the upper cervical region of the spinal cord, for the muscles in the adult that produce similar movements are innervated from that region. The cells of the spinal accessory are probably among the first to take part in the activity, for both the sensory and motor fibres develop precociously (Windle, 1931) and it is one of the first peripheral nerves to acquire extensive medullation (Windle *et al.* 1934; Barron, 1936).

Though considerable attention has been directed to the identification of the particular group of sensory fibres through which the first reflex activity can be aroused in ontogeny, the results are by no means in agreement. In some of the forms studied (rat, guinea pig, sheep; see Table III) the first reflexes have followed stimulation of the nerves distributed to the neck and the forelimb. These differences should not, however, be taken to indicate that the anatomical elements of the reflex arcs develop first in the lower cervical region of the cord in some animals and in the hind brain in others. In fact, so general is the law of cephalocaudal differentiation in mammalian development that it would be surprising, indeed, if reflex arcs were not completed anatomically first in the upper cervical segments and only later in the brachial, thoracic and lumbar regions of the spinal cord. Angulo (1936*b*) claims that the first somatic sensory fibres to make central connexions in the rat are distributed peripherally in the trigeminal nerve. Presumably sensory fibres of the second, third and fourth cervical nerves make their central connexions in that order in development, for Windle (1931) has pointed out that in the cat embryo the development of collaterals from the dorsal funiculus proceeds in a cephalocaudal direction. Certainly careful examination of the sensory collaterals of the dorsal funiculus in sheep embryos demonstrates that the more cranial ones penetrate deeper into the grey matter than those given off in more caudal segments (Barron, unpublished). Why then is this cephalocaudal differentiation of the nervous system not apparent in the physiological responses of the embryo, and why is there no

Table III. *First reflexogenous zones to direct stimulation*

Animal	Foetal age	First reflexogenous zone	Movement	Authors
Rat	16th day	Side of snout	Lateral flexion of head away from stimulus	Angulo (1932)
Rat	16th day	Corner of mouth, shoulder and side	Involved neck and fore-limbs	Raney & Carmichael (1934)
Rat	16th day	Snout	Lateral flexion and extension of head	Windle *et al.* (1934)
Rabbit	15–16 days	Nose, head and neck regions	Lateral flexion in the neck and upper trunk	Pankratz (1931)
Cat	23rd day	Head, shoulder and paw	Reaction of foreleg	Coronois (1933)
Cat	28th day	Nose and ears, to a lesser degree other points on the head	Extension, lateral flexion and rotation of head	Windle & Griffin (1931)
Guinea pig	31 days	Ear	Flexion of neck, raising of forelimb	Carmichael (1934)
Sheep	34th day	Face	Head extension	Barcroft *et al.* (1936)
Man	8 weeks +	Face	Flexion of body, involving muscles of limb girdle	Hooker (personal communication)

agreement as to which group of sensory fibres when activated produce the first reflex response in ontogeny? Reasoning upon the evidence from morphological studies we might have expected that stimulation of the sensory fibres of the trigeminal would produce the first reflexes. These initial reactions would be followed by reflexes via the first, second and third cervical nerves in that order and so on caudally along the body.

The embryonic anterior horn cell undoubtedly has a high threshold as has other immature tissue (Soltmann, 1877; Westphal, 1886; Banu, 1922). To excite such an immature cell would require a series of impulses arriving over a small number of sensory fibres (summation) or a single volley of impulses over a large number of fibres. The second result is most easily obtained by the stimulation of a large sensory nerve, and hence one might expect the first reflexogenous zones to be those in which large nerve trunks are distributed. Such areas are to be found on the face where fibres of the maxillary division of the trigeminal may be excited directly and in the limb areas where the sensory fibres are combined into the cords of the plexuses.

Here we must pause and remind ourselves that the size of the sensory nerve roots entering the brain or spinal cord has been shown in amphibia (Detwiler, 1920), birds (Hamburger, 1934), and in the mammalia (Barron & Barcroft, 1938) to be determined by the amount of mesoderm into which they are distributed peripherally. The greater the amount of mesoderm, the larger the sensory nerve and the larger the number of collaterals that enter the spinal cord at the level at which the sensory roots enter. The relationship between the number of these collaterals and the motility of an embryo has been studied by Windle & Baxter (1936). In motile embryos of the same size and litter there are 80% more collaterals penetrating into the grey matter in the brachial levels than in non-motile embryos. The number of these collaterals is due to the presence of the limb. Hence we see that the presence of a limb might allow a reflex to be demonstrated through a region of the spinal cord that was actually behind in its structural differentiation, regions in which no functional reflex arcs can be established.

In this connexion Windle and his co-workers (Windle *et al.* 1935; Barcroft *et al.* 1936) have been able to demonstrate what appear to be reflex contractions of muscles moving the head, the forelimb and the hind limbs respectively at a stage in ontogeny when the muscles lying topographically between these three groups cannot be similarly activated. These contractions of muscles are best seen immediately after delivery of the embryo in the intact amniotic sac. They can be evoked by tapping on the sac, thus transmitting pressure through the amniotic fluid to the embryo. The evidence that these movements are reflex, involving a sensory and a motor neurone, is based not only upon the facts that the musculature contracts after a perceptible delay and that the mechanism fatigues readily, but upon the anatomical studies of Windle and his collaborators as well. At the time these movements occur collaterals have been given off the dorsal funiculus of the spinal cord and have penetrated into the grey matter, though direct connexions with anterior horn cells have not occurred. Though these movements have not been universally accepted as reflexes (Angulo, 1935; Barcroft & Barron, 1937*a*), their isolated character has led Windle (1936) to consider the probability that mammalian somatic behaviour has its genesis in simple non-integrated, spinal type reflexes in accordance with the second theory presented at the outset.

The order in which reflexogenous zones or nerves appear in intrauterine development, at least under the conditions in which they have been studied, depends upon so many physiological factors that the available results are difficult to interpret in terms of the behaviour problem, *if*, indeed, they have any bearing upon it. Unlike the free-living urodele, Amblystoma, studied by Coghill (1929), the mammalian embryo in its normal condition in the uterus is isolated during the period under consideration in an environment whose constancy has been emphasized by the investigations of Barcroft (1938) and his school. The Amblystoma exists in a changing environment to which it is continually adjusting itself as it develops, but the mammalian embryo on the other hand has no need for mechanisms to adjust it to changes in its external environment until it gives up its intrauterine existence. The mammalian embryo can and does adjust itself to changes in its internal environment (under normal conditions these are of a chemical nature), but there seems little reason to believe that the reactions of an embryo to a stimulus so unnatural as a pressure transmitted to it by a blow on the amniotic sac or the direct pressure of a glass rod are in any sense an "adjustment" to an external change or have any real relationship to its behaviour when the foetus finally prepares for extrauterine life. To assume that these intrauterine responses are related to adult behaviour may be similar to assuming that the structure of a caterpillar would reveal the genetic characters of the behaviour of a butterfly. The foetus is being constructed *in utero* for an external existence and though its reactions to artificial stimuli can and have revealed a great deal about the manner in which this construction progresses, the behaviour of the foetus as an "individual" cannot be said to begin until it is launched in the external world and begins to lead an independent existence.

(2) *The spread of the motor response*

Though these first reflexes may have no real bearing upon the problem of adaptation to external environment, they do reveal that the first effective sensory impulses activate muscle fibres in a very circumscribed region of the body. Whether this limited response in the muscular system is due to the absence of more extensive anatomical connexions within the central nervous system or to the relatively low excitability of the structural elements involved has not been determined. However, as development does advance and more neuronal connexions are made within the brain and spinal cord the responses to sensory stimuli for a time grow, involving muscles more and more remote from the site of the stimulus. The manner in which this response spreads depends in a great measure upon the central connexions of the sensory fibres that are stimulated, and the growth of the connector neurones upon which they end. Hence the muscular response that follows stimulation of a given sensory nerve at any time during ontogeny depends upon at least two factors:

its central connexions and the state of differentiation of the connecting elements, linking it with the motor system.

Some sensory nerves have central connexions with the caudal conducting bulbospinal system of fibres whose cells lie in the hind brain. Among these sensory nerves may be mentioned the maxillary and ophthalmic branches of the trigeminal (see Kappers *et al.* 1936) and in mammals some of the sensory fibres of the uppermost cervical nerves. By virtue of these connexions, the activity these sensory fibres produce might be expected to depend upon the extent to which the bulbospinal system was developed.

Stimulation of the maxillary nerve early in ontogeny is followed only by a slight movement of the head due to a contraction of muscles supplied by upper cervical nerves. A few days later the same stimulus produces a response which involves not only the muscles of the head but those of the forelegs as well. Still later muscles of the trunk, hind legs and tail are incorporated in the response in that order. The caudal spread of the response does appear to indicate the progressive caudal growth of the fibres of the bulbospinal system. The muscles reacting appear to be represented in the adult by the extensors and abductors of the limbs and by the dorsal longitudinal muscles of the trunk, for the response is of the extensor type. In some forms, the sheep for example, connexions are established between the sensory fibres of the maxillary nerve and the motor neurones to the musculature moving the tail before any functional link is established between the sensory fibres of the maxillary and the motor nuclei supplying the muscles that open the mouth or move the tongue. The sequence of events is much the same when the ophthalmic division of the trigeminal is studied. These two nerves supplying as they do the anterior or future advancing pole of the body apparently acquire connexions with the locomotor apparatus of the animal in ontogeny before they are linked with the visceral mechanisms represented by the mouth and tongue. A similar caudal spread of the motor response that follows stimulation of the maxillary nerve has been observed in rats by Angulo (1932) and in man by Hooker (1936).

Other sensory nerves primarily related to the bulbospinal system of connector neurones are the fibres of the vestibular division of the eighth cranial nerve and dorsal root fibres of the uppermost cervical segments that end peripherally in the small post occipital muscles. These fibres distributed to the post occipital muscles and to the cranial-most vertebral articulations produce motor effects before those in the vestibular portion of the eighth at least in the cat (Windle & Fish, 1932) and sheep (Barcroft & Barron, 1939). The position of the head of the sheep foetus very early in ontogeny appears to dictate the position of the forelimbs. In older foetuses not only is the position of the forelimbs determined by the position of the head, but the attitude of the trunk as well. Later still the hind legs assume attitudes determined by the position of the head. Finally, the orientation of the eyes in the orbits is brought under the control of the same mechanism. Since the labyrinths have been shown not to be responsible for the regulation of these postural attitudes (Barcroft & Barron, 1939) the inference is that these attitudes are the embryonic expression of the tonic neck and righting reflexes described by Magnus and de

Klejn (see Magnus, 1924) in adult mammals. These reflexes are initiated by the discharge of sensory impulses via the sensory fibres of the nerves distributed to the small post-occipital muscles and the atlanto-occipital articulation. After the manner of the maxillary and ophthalmic branches of the trigeminal these sensory fibres progressively gain control over the postural muscles of the body as fibres of the bulbospinal system make connexions with motor neurones situated more and more caudally.

In contrast to the generalized responses evoked by stimulation of the ophthalmic and maxillary divisions of the trigeminal and the sensory fibres to the small muscles of the post-occipital region are those aroused by the mandibular branch of the trigeminal and the sensory fibres to the muscles of the limbs. Stimulation of the buccal branches of the mandibular is followed first by opening of the mouth and later in ontogeny by tongue movements as well. Touching the tongue, thereby stimulating the ends of fibres of the lingual nerve, is followed first by closure of the mouth and only later in development by sucking movements following the closure. Never during intrauterine development does the response from either of these nerves spread, in foetal sheep, beyond the visceral and the branchial musculature (Barcroft & Barron, 1939).

Similar local muscular reactions are all that ever appear, during intrauterine life, to follow stimulation of the sensory nerves to the muscles of the limbs of sheep embryos and foetuses. Flipping of the limbs, thereby stretching some of the muscles, and presumably exciting their sensory nerves, produces at first only the contraction of the muscles in the proximal segment of the limb, but in older foetuses the response spreads to include all of the extensor muscles and the adductors in the limb. When, in still older foetuses, the response spreads beyond the confines of the limb stimulated it crosses the middle line to involve the opposite limb. No generalized reactions, however, appear to follow excitation of any proprioceptor fibres in either fore or hind limbs. It is interesting to note in passing that the sensory fibres to muscles appear to be the first system of somatic sensory nerves to be functional in the normal way (Barcroft & Barron, 1939). Certainly they are the first fibre system to become medullated (Anderson, 1898; Barron, 1936). If this supposition regarding their function is supported by direct experiment, we will be presented with another departure of ontogenetic from the course of phylogenetic development; in the latter case the exteroceptor system appears first.

These early responses evoked by the stimulation of a sensory nerve appear to be related, therefore, to the nature of the central connexions of the sensory fibres with the internuncial system of neurones and to the extent to which the internuncial system has developed and connected with the motor system. Each sensory fibre appears during development to establish broader and broader connexions with the motor mechanisms, by the dual expansion of the internuncial system and its own terminations with that system. Those sensory nerves primarily related to the trunk and body through the longitudinal conducting systems when stimulated produce generalized reactions. Those related to the local or segmental connector neurones normally when excited produce relatively localized reactions. If one of these systems of connector neurones should develop functionally in advance of the other, the present experimental methods could not be relied upon to demonstrate which is first.

Structurally all sensory nerves must go through this process of developing and expanding central connexions, but the process of their expansion may not always be physiologically demonstrable. Those sensory nerves that become functional relatively late in gestation rarely provoke more than a very limited response. The same sensory nerves when studied in different embryonic forms do not produce a similar series of responses during development. We have indicated earlier that the motor response to a sensory volley depends not only on the central connexions of the sensory nerve but upon the functional state of the central nervous system. We can now consider how in its physiological state the central nervous system is altered during development.

(3) *The segregation of muscle groups*

The earliest motor responses aroused by sensory stimulation transmitted via either of these connector or internuncial neurone systems have the appearance of being fixed; that is, they are graded or modified only by the strength of the stimulus applied to the sensory nerves. They retain this character for a short time in ontogeny. Soon, however, they lose this automatic predictable character and their magnitude depends upon factors other than the intensity of the stimulus to the sensory nerve. To illustrate these statements: By the fortieth day of gestation, the motor responses to stimulation of the maxillary nerve of the sheep foetus have spread, after the manner described above, to include the musculature of the trunk, tail and the fore and hind limbs. During the next few days of gestation the response to a single stimulus is no longer as extensive as earlier and as development advances becomes less and less extensive. The muscles drop out of the response in approximately the reverse order to that in which they were incorporated, until by the forty-fifth day a single stimulus applied to the nose is followed only by head extension or lateral flexion. Repetitive stimuli can, however, be relied upon to produce the more extensive response seen earlier. The former pathways appear still potentially present, but a certain "resistance" to the spread of the response has been introduced. In the same way the righting responses are submerged. Still using observations made upon the sheep embryo to illustrate this restriction of the motor responses we may consider those induced in the limbs. Between the forty-fourth and forty-seventh days of gestation responses of the type described above can first be aroused by "flipping" the forelimb. By the fiftieth day the response has spread from the stimulated limb to include muscles in the opposite one. Ten days later, however, the response has been reduced to the contraction of the adductor muscles that were the first to respond. This restriction of response is apparently a general phenomenon, for Minkowski (1921) and Bersot (1921) have described in detail the spread and ultimate restriction of the muscular response to stimulation of the plantar surface of the feet of human foetuses delivered by Caesarian section.

The expansion or spread of the motor response has been shown above to be associated with the growth of the central connexions of the sensory and internuncial neurones and may also be related to the decrease in the resistance of the individual synapses encountered, though as yet no direct evidence on this point is available. The restriction of the response is certainly not due to a reversal of these processes of development. The restriction of the motor responses appears rather to be the result of the same factors that are concerned in its expansion.

During the early stages of development the forelimbs move as an integral part of the generalized response to a single stimulus evoked via the sensory nerves to the head, in all the forms so far studied. Gradually, however, the limbs cease to take part in the activity. They become "segregated out of the total pattern" as Coghill (1929) has expressed it. Repetitive stimuli to these same sensory nerves usually bring out the total or generalized response in which the limbs take part. A resistance has developed in the pathway from the nose to the limbs that can only be overcome by repetitive stimuli. How this resistance is produced has not been determined, but the degree to which it has developed can be directly related with the structural appearance of the spinal cord at the brachial levels. In the sheep (Barcroft & Barron, unpublished) at the time the limb begins to acquire its independence, collaterals may be traced from the posterior funiculus of the spinal cord to the anterior horn cells of the same side and as the independence of the limb increases these collaterals grow in number and extent. Apparently the development of the direct local reflex mechanism is in some way associated with the release of the limb from the general activity. Doubtless this is but a single instance of a relatively general phenomenon whereby the activity of the foetus is functionally broken up or segregated into units through the ingrowth of sensory fibres from the periphery.

Unfortunately we have little direct evidence as to the mechanism by which the collaterals ending on the anterior horn cells produce this effect. Since the currency of the nervous tissue is in impulses we might imagine that impulses arriving via the sensory nerves to the limb inhibited the anterior horn cells from participating in the generalized response. While no crucial evidence is at hand to indicate the existence of such impulse discharges at this stage, certainly in the adult central nervous system activity at one point cancels out activity initiated in another, and there is no reason to believe that this is impossible in the embryo.

Once this segregation or "restriction" process has begun it is obvious that generalized responses are no longer easily evoked. Those sensory nerves that impinge upon the central nervous system early in ontogeny alone possess the capacity to arouse general activity. The later in ontogeny a sensory nerve makes functional connexions with the central nervous system, the more restricted the motor response it elicits when stimulated may be expected to be.

This point can be illustrated by a comparison of the motor effects produced by the stimulation of the sensory nerves of the skin in embryos and foetuses of different species. In the rat (Raney & Carmichael, 1934) the first movements of the embryo in response to tactile stimuli of regions other than the head appear during the sixteenth day, i.e. relatively early in ontogeny. In general terms this tactual receptivity, as judged solely by the production of motor effects, appears to develop in cephalocaudal, proximodistal direction. The first motor responses elicited in the rat are diffuse and generalized with relation to the skin area stimulated. In very young rat foetuses, for example, no matter where they are stimulated, neck or trunk bending results. Touching the lower surface of the fore paw of a foetus seventeen and a half days old is followed by a generalized response involving the trunk and limb. As the birth time approaches, however, greater specificity of response in relation to stimulation occurs. Thus segregation and the restriction of the motor responses can be functionally demonstrated by stimulation of the nerves to the skin.

Compare these responses with those observed on the sheep foetus. Not until about the middle of the long gestation period (*c.* 150 days) can true exteroceptive responses be elicited from the body or limbs of sheep foetuses (Barcroft & Barron, 1939). Then stroking the distal segments of the fore limb may result in flexion and withdrawal of the leg but never in a generalized reaction. Stimulation of the dorsal surface of the foot is followed by extension and abduction of the toes. All of the responses elicited via the sensory nerves to the skin appear in sheep to be localized from the very beginning after a manner attained in the rat rather late comparatively in development. The generalized reaction stage, so clearly described in the rat by Raney & Carmichael (1934) and Angulo (1932) never occurs in the sheep. At the time these sensory fibres from the skin in the sheep establish their first functional connexions with the spinal cord a sufficient number of sensory fibres from the muscles have already arrived and produced some of the effects of segregation and restriction.

But the motor neurones themselves are subject to other forces that result in more restricted, more localized responses of the muscular system. In passing from embryonic stages to adult, or from phylogenetically low to higher forms there is a definite tendency for the motor cells to group themselves into longitudinal columns which appear to be associated with the innervation of certain functional groups of muscles (Woodburne, 1939). By segregation in space these cells must restrict the nervous influences acting upon them. For this reason considerable interest is attached to the appearance and maturation of these columns. Thus far no detailed study of this segregation correlated with activity has been published though some are in progress. In sheep no definite division of the anterior horn cells into columns can be seen until just prior to the twenty-fifth day. The medial and lateral cell masses are distinguishable at thirty days, but individual cell columns within these masses, though present, are not easily made out until after the thirty-sixth day. The cell columns are therefore in the process of segregation at the time the first reactivity of the embryo occurs. By the thirty-ninth day the fundamental cell columns are laid down. As development advances and movements become more localized there is an obvious augmentation and condensation of the columns as new cells appear in them—in many instances by migration into them from surrounding grey matter.

Thus far we have considered the segregation of muscle groups solely as the result of the growth and the functional differentiation of neural elements. The recent experiments of Weiss (1936) have, however, demonstrated that in the amphibia a certain amount of segregation and individuation in the activity of the muscles must be ascribed to influences which emanate from the musculature to the motor neurone. These muscles, Weiss has demonstrated, "modulate" the anterior horn cells supplying them and hence determine in part their receptivity to neural influences reaching them centrally. Coordination of movement therefore depends in these forms to some extent upon the state of differentiation of the muscular system. How far this effect of "modulation" is responsible for some of the segregation of motor responses during ontogeny in mammalian forms remains to be determined, but the existence of such a process must be regarded as possible.

(4) *General inhibition*

At the same time that the local segmental sensory fibres are making central connexions, fibres are reaching the spinal cord from higher centres of the nervous system in large numbers. Though these descending fibres undoubtedly contribute to the processes of functional segregation going on among the motor elements of the spinal cord, their most pronounced effect is upon the motor system as a whole. Their first obvious effect is to alter the character of the movements of the foetus and later to inhibit them almost completely until birth releases them again. The individual stages of this process are best seen in a slow-growing foetus with a long gestation period.

Until about the forty-second or forty-third day of intrauterine life the movements of the sheep embryo, both "spontaneous" and induced, resemble muscle twitches. The limb or head is brought sharply into a new position by the contraction of muscle fibres and then immediately returns to the resting attitude. There is never any obvious tendency for the embryonic muscles to remain contracted. Beginning about the forty-second or forty-third day, however, there is an increasing tendency for the moving parts—the head or limbs—to remain in any newly acquired position. In the earliest stages this amounts to little more than a hesitation of the moving part before it returns to its original position. This tendency increases until by the forty-seventh day the limbs and head are obviously held in new attitudes and positions by muscular effort. Since, as we have seen earlier, this property is not possessed by the muscle in the absence of nervous stimuli, it seems reasonable to assume that whereas earlier the anterior horn cells supplying the contracting muscle were discharging a single volley, in the later stages, those in which the muscular contractions were maintained, the anterior horn cells were discharging impulses repetitively. Superficially the foetus appears to have been converted during its development between the forty-third to forty-seventh day, from a bulbo "spinal" animal into one possessing at least some functional mid-brain structures. The evidence that this alteration in the character of the movements is due to the onset of function in some structures in the mid-brain comes from two sources, i.e. from stimulation and extirpation experiments.

The sheep foetus is large enough by the thirty-eighth day of gestation for faradic currents to be easily applied to the dorsal surface of the mid-brain or to the cortex of the cerebral hemisphere. The earliest movements that follow faradization of the dorsal surface of the mid-brain occur on the forty-first day. The legs, both fore and hind, are flexed on the body. This response does not last as long as the stimulus is applied, for the central mechanism concerned fatigues to prolonged stimulation very rapidly. By the forty-third day the movement elicited from the mid-brain has begun to take on another character. After the same kind of stimulation, the legs of one side extend and those of the other side flex. During the time the stimulus is applied the pattern may reverse itself in a clonic fashion. The legs on one side flex and extend rhythmically. With further development the response is crystallized and by the forty-fourth to forty-seventh day stimulation of the mid-brain is followed by a flexion of the limbs on the side excited and extension of those of the opposite side. At the same time the long muscles of the back of the same side contract so the neck and body are curved toward the side stimulated. This attitude, maintained by prolonged muscular contraction while the stimulus is applied to the mid-brain, is not unlike that described by Hinsey *et al.* (1930), as following stimulation of the tegmentum of the mid-brain in cats. These authors suggested that the responses they observed were conducted caudally by a rubro-reticular element finally reaching the spinal end by the reticulo-spinal pathway.

During this same period (forty-fifth to forty-seventh day) the responses of the diaphragm and the other muscles concerned in respiration undergo a considerable change. In the young foetus each stimulus was followed by a single contraction of the diaphragm. As the contractions of the muscles of the limbs are sustained for longer and longer periods, so the rhythmic contractions of the diaphragm continue longer and longer. The respiratory movements lose the "gasp-like" character they possessed earlier and by the fiftieth day may vary both in rate and in amplitude. This gradual alteration in the character of these movements appears also to be associated with the onset of function of cells in the mid-brain region, for by the forty-seventh day faradization of the mid-brain, as we have seen, increases the effort of the respiratory muscles.

This takes us up to about the fiftieth day of gestation. Within the next few days—the fifty-second to the sixtieth—the activity of the foetus becomes more and more restricted. Finally a stage is reached when the foetus delivered for study assumes an attitude not unlike that of a decerebrate preparation. The head, the four limbs and tail are all rigidly extended. Windle & Griffin (1931) first described such an attitude in cat foetuses and aptly likened it to "stretching". In the sheep foetus this attitude is maintained in the absence of any obvious external stimulation except that attendant upon its exposure. The resemblance of the foetus to a decerebrate animal immediately suggests that some of the same functional and structural elements are concerned in both cases.

During the next ten-day period in gestation—between the sixtieth and seventieth days—these former postural attitudes are suppressed. The foetus now passes with advancing age into a state of lethargy and inactivity. Any stimulation, even if intense, elicits but a feeble local reaction. These gradual changes in the character of the activity of the sheep foetus are accompanied by the appearance of certain reflexes which give a hint as to the nature of the structural developments that make them possible. As early as the forty-first day of gestation the tonic neck reflexes on the limbs can be demonstrated. Bending the head downward is accompanied by flexion of the forelegs and extension of the head by extension of the forelegs. Rotation of the head is accompanied by extension of the limb on the side toward which the nose is turned and flexion of the opposite leg. Compensatory movements of the eyes to movement of the head have been observed on the forty-ninth day. Head righting on the body occurs usually about the forty-seventh to the fiftieth day. Magnus (1924) has demonstrated that in adult animals the region of the brain stem between the level of entry of the vestibular nerve above and the upper cervical cord below contain the structures essential for the execution of the tonic neck and labyrinthine reflexes except righting. The centres essential to the righting reflexes are in the mid-brain. Taken together these data suggest that the changes in the activity from jerky movements to sustained, from a gasping type of respiration to one easy and continuous, might be associated with the functional maturation of structures lying along the neuraxis from the upper cervical cord to the upper end of the mid-brain.

This inference receives support from experiments in which transections and mid-sections of the brain stem were made in a series of foetuses (Barcroft & Barron, 1937*b*). These experiments demonstrated that only the gasping type of respiratory movement persists if the brain stem is transected at any age behind the level of the entrance of the eighth nerve. Once continuous respiratory movements have developed ontogenetically the section must pass anterior to the pons if the activity is to be retained unimpaired. The inhibiting forces which suppress respiratory activity from about the fifty-fifth day onward are located in the mid-brain and the regions lying more anterior. The most pronounced release is produced by sections that pass just through the region of the posterior colliculi. The inhibiting influences appear to decline the more forward they lie. The mid-brain depresses respiration more than the basal ganglia, the basal ganglia more than the cortex, but all of these regions, if removed, appear to produce some release.

The effect upon the character of the movements of the limbs is similar. A section which passes posterior to the entrance of the eighth nerve leaves the foetus—whatever its age—capable of only jerky movements. If the section lies forward of the eighth, the movements if once sustained remain so after the operation. The farther forward the section is placed, the more prolonged they may be. The inhibition which finally suppresses the decerebrate attitude described above appears to be associated with structures lying ahead of the mid-brain. In as much as decortication of a foetus produces no obvious release the inhibitory centre may be located in the basal ganglia.

Putting all this in another way we may infer that the earliest activity of the foetus took place in structures in the spinal cord and lower medulla. Between the forty-first and the forty-seventh days the region of the pons began to influence the spinal mechanisms, to be followed during the next ten days by structures in the mid-brain. Finally toward the sixtieth day the activity of the foetus was inhibited by the functional maturation of structures located in the basal ganglia.

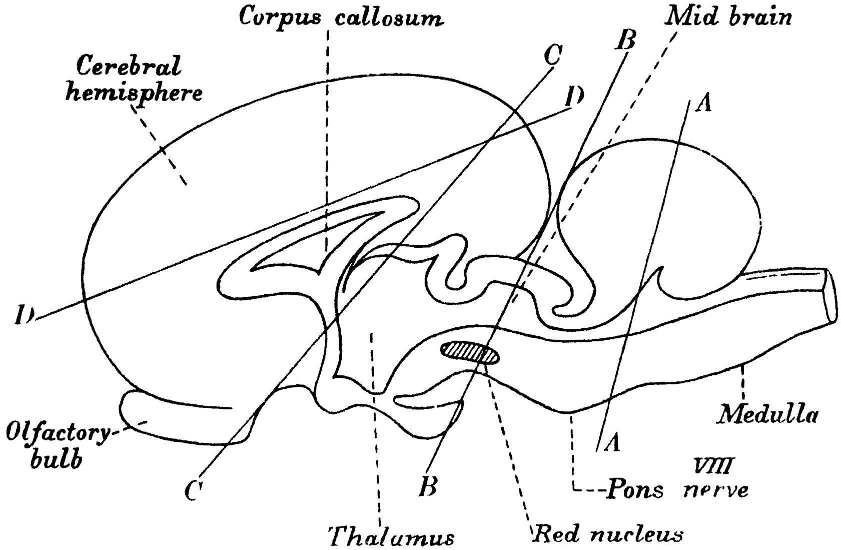

Fig. 1. A diagram of a foetal sheep's brain showing the levels of transection used in the analysis of the development of the influence of higher centres upon spinal mechanisms. Transection at A, all movements jerky; B, movements sustained; C, sustained movements inhibited; D, no influence on spinal "centres".

These observations upon the sheep foetus may be paralleled by the studies of Minkowski (1928) upon the development of the plantar reflex in human foetuses. Minkowski found by extirpating portions of the foetal nervous system that the human foetus is, during the third and fourth months of foetal life, essentially a bulbo-spinal preparation. In mid-foetal life—fourth to sixth months—it develops into a tegumento-bulbo-spinal mechanism by the functional maturation of structures anterior to the bulb. At the same time that the tegumentum becomes functional there is a marked restriction in the reactions of the foetus to external stimuli. The last stage of human foetal life is marked by the onset of function in the striatal and cerebellar systems. This Minkowski has termed the pallido-rubro-cerebello-tegmento-spinal stage.

A consideration of other available studies on the development of foetal motility, particularly those of Angulo (1932) and Windle & Griffin (1931), leaves little doubt that these phenomena illustrated in the sheep and the human foetus are general phenomena. The functional differentiation of the structures in the mammalian brain concerned with the regulation of muscular activity begins first in the hinder end of the mid-brain and spreads slowly forward. Each new addition appears to inhibit the activity of mechanisms already laid down. This phenomenon is not without general significance, for it emphasizes the fact that a point somewhere near the junction of the spinal cord and the medulla is the first to differentiate and that from this point differentiation proceeds caudally and cranially, at least so far as motor mechanisms are concerned. Further, the mode in which the new functional centres of the brain develop only serves to stress the reality of the phenomena that are exhibited by adult mammals following the destruction of the "higher" centres. Far from being "pathological" many of the phenomena released are natural and normal, and possess a previous history in the nervous system whatever may have been the direct cause of their later emancipation from dominating "higher centres".

In keeping with this principle that the higher structures in the nervous system become functional late in ontogeny are the observations on the functional maturation of the cortex. No spontaneous electrical activity can be recorded from the cortex of the rabbit until the fifth day after birth (Sjöstrand, 1939; Kornmüller, 1935[1]). Faradization of the rabbit motor cortex first evokes movements on the tenth day post-partum (Soltman, 1877), but the motor cortices of the sheep (Barcroft & Barron, unpublished) and the monkey (Hines, 1937) are already excitable before birth. Spontaneous electrical activity may be recorded from the cortex of a kid on the day it is born (Sjöstrand, unpublished); yet intrauterine decortication has no obvious effect upon the activity of a sheep foetus with intact placental circulation. The cortex appears to be in a functional state structurally, but exists (see Barcroft, 1938) *in utero* in an environment so poor in oxygen that its potential activity cannot be expressed though the oxygen supply appears to be adequate to allow for structural differentiation. Other portions of the nervous system as well may be held in a comparable state of "suspended animation", but of their existence we have as yet no knowledge. The simple fact, however, that some parts of the foetal nervous system are able to function in the environment provided by the mother and that others apparently are not, does emphasize the fact that cells of the embryonic brain do differ considerably with regard to their oxygen requirements.

These differences can be illustrated by the events that follow asphyxiation of a human foetus (Minkowski, 1928) or a sheep foetus (Barcroft & Barron, 1937*b*). Imagine that we have just delivered from the uterus an eighty-day sheep foetus for observation. If the umbilical circulation be interrupted so that the foetus is no longer receiving oxygen from or giving up carbon dioxide to its mother, the foetus slowly but certainly dies, for it cannot at this stage ventilate its own lungs. But during the time it remains alive its activity undergoes several changes. Though drowsy and lethargic when first seen, after a few minutes the foetus will assume the stretching or decerebrate attitude. A few minutes later this attitude will have passed off to leave the foetus capable only of sustained muscular contractions. Still later the movements will have become jerky. As the jerky movements appear irradiation becomes more and more marked so that eventually tapping the foetus anywhere on the body is sufficient to provoke a generalized reaction involving the trunk and all of the limbs. These jerky movements represent the last stage of activity before the foetus becomes moribund.

These results are essentially similar to those of Minkowski (1928). The effect of progressive asphyxiation on the Babinski reflex in the human foetus is what one might expect to follow a series of transections of the brain stem from above downwards. Asphyxiation first removes the influence of the basal ganglia upon the reflex, later the influence of the tegmentum, leaving the reflex as a purely spinal mechanism freed from the higher centres.

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[1] *Biological Reviews.*

Clearly these functional centres in the brain that influence the spinal reflex mechanisms differ during intrauterine life in their susceptibility to asphyxia. Those centres that develop last are affected first. This phenomenon appears to be a general one, for Windle & Griffin (1931) have pointed out that in the cat foetus asphyxiation abolished those movements that were acquired last in ontogeny first and that the older components appeared more resistant and persisted longer. Windle & Becker (1940) have also stressed the fact that local responses are thrown out and mass reactions are increased by partial anoxemia, especially in foetal cats during the first half of the gestation period. Angulo (1933), as mentioned earlier, has suggested that newly developed nervous mechanisms have the highest metabolic gradients, are therefore most susceptible to asphyxiation, and hence are excited first by the inevitable accumulation of metabolites in the blood. The present body of information, however, is in but partial agreement with these suggestions, for though the most recently developed centres do appear to be those first affected by asphyxiation, instead of being excited their activity appears to be inhibited. When postulating that accumulated metabolites in blood are able to excite nerve cells directly, it is well to consider the caution of Barcroft (1934): "It is much easier to conceive of CO_2 as paralysing a nervous process than as stimulating it".

The effects of asphyxia not only emphasize the differential susceptibility of the cells in the central nervous system but the intimate relationship that exists between the activity of a foetus or embryo and its blood supply. Since the blood supply under experimental conditions tends in varying degrees to be poorer than normal, so the embryo tends usually to be more active outside the uterus than inside unless it is moribund. So obvious is this relationship, it is astonishing that so little exact data with regard to the amount of oxygen in the foetal blood and the levels of the foetal and maternal blood pressures have accompanied description of activity. Such exact data are needed correlating foetal activity with the oxygen and carbon dioxide levels together with the hydrogen ion concentration of the blood bathing the foetal brain. One such correlation has already been presented by Barcroft *et al.* (1940) who have demonstrated that the oxygen level of the blood taken from tontanelles of foetal sheep aged one hundred days and upwards just after the respiratory movements appear following occlusion of the umbilical cord is remarkably constant. The level at which the release of the respiratory activity takes place drops from about 25 % saturation at one hundred days to about 15 % at term.

All of the data so far considered bear upon the motor mechanisms of the foetus aside from isolated facts with regard to the sensory nerves. As this data affords little support for any postulated endogenous activity, it seems impossible to escape the conclusion that part at least of the changing character of the activity of the foetus during gestation is due either to a change in the amount or the nature of the sensory information arriving at the central nervous system.

The ontogeny of the sensory system is almost completely unknown. Though there are several admirable accounts of the histogenesis of sense organs (Hewer, 1935; Tello, 1917; Elwyn, 1929), we have no exact knowledge as to how their structural development alters the type of sensory response in the fibre leaving them for the central nervous system. The results of Dickson (1940), who has studied the histological development of muscle spindles and motor end plates in the intercostal muscles and the diaphragm, do give us a hint as to the effect of increased structural complexity of muscle spindles upon the motor responses. These results demonstrate that the time at which muscle spindles are first differentiating in the respiratory muscles coincides roughly with the onset of sustained movements in the fifty-day period. A summary of Dickson's results appears in Table IV. These data have also been introduced into the scheme, Fig. 2, to illustrate the correlation.

Table IV. *Dickson's histological observations summarized*

Age Days of gestation	Sensory endings	Motor endings
40	No spindle found	Undifferentiated
47	Very early spindle	Doubtful
66	Simple definite spindle	—
80	Free interfascicular ending	Typical motor distribution
92	Very complexed spindle	Typical motor distribution
137	Very complexed spindle	Early motor end plate

The vestibular part of the ear may also contribute to the sensory background in the fifty-day period, for the sensory endings in the ear are already well differentiated by the forty-fifth day and I have noticed a definite decrease in the tone of the limbs after destruction of the labyrinths in the foetus of about sixty-five days. This of course suggests that some sensory information is reaching the brain stem from the macula of the utricle. Little more of value can be added now, but these remarks will serve to emphasize how little is known and perhaps serve to encourage a detailed study of the ontogeny of the sensory system using the technique of Adrian and Matthews.

We have thus far considered six aspects of the functional development of neuromuscular mechanisms during the intrauterine life of embryos and foetuses: the period of differentiation or inactive period, spontaneous activity, the spread of motor responses to sensory stimuli, segregation of muscle groups, general inhibition and the influence of the environment. In slow-maturing foetuses these aspects might appear to represent successive stages in ontogeny, each one merging into the next. But in rapidly growing foetuses and embryos these stages do not follow one another but appear to take place almost simultaneously. For example, an analysis of Angulo's studies on the rat indicates that the embryo is inactive until the fifteenth day. During the sixteenth and seventeenth days the movements are unsustained and hence it might be considered a bulbospinal animal. By the eighteenth day the tegmentum has become functional as evidenced by the sustained muscular contractions that occur and the fact that asphyxiation now alters the activity. Until

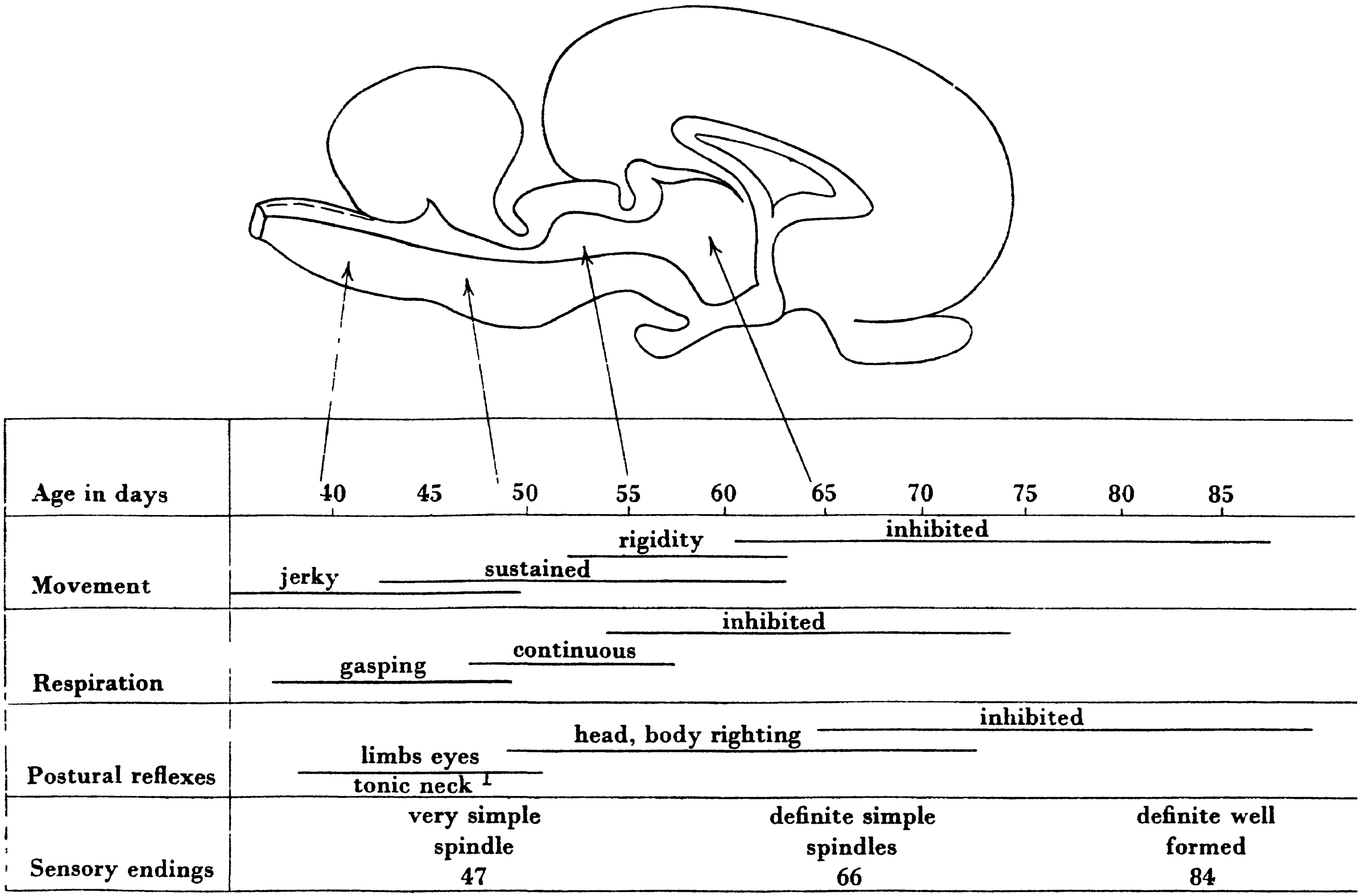

Fig 2. A schematic representation of the gradual change with time in the activity of the sheep foetus together with the brain regions concerned and with the differentiation of muscle spindles.

birth three or four days later inhibition of activity is gradually increasing, presumably through the influence of structures in the forebrain—the diencephalon and the basal ganglia. All of the events seen to take place over a period of two months in the sheep are telescoped into a single week by the rat. The influence of the peripheral sensory field appears to make its impression on the cord at the same time that inhibitory tracts are reaching the same motor horn cells. Such a comparison between the development of the sheep and the rat only serves to demonstrate the difficulties experienced when one attempts to make detailed comparisons of the activity between one embryonic form and another. A reasonable doubt might be entertained that the responses are ever directly comparable. Little wonder that students in the behaviour problem have found difficulties in comparing their data.

And this consideration brings us back to the original point of departure in our discussion of these mechanisms: the behaviour problem. Which of the two theories is the correct one? The observations summarized here certainly do not afford a solution, but they may assist others to an earlier one.

V. SUMMARY

Some of the literature pertaining to certain aspects of the functional development of the neuromuscular mechanisms of mammalian embryos and foetuses has been reviewed.

There appear to be some features that are common to all of the animal forms so far studied. In the early stages of development the embryos are inactive. Toward the end of this inactive period—the length of which varies from one form to another—the skeletal musculature begins to respond to direct faradic stimulation. The nervous system is related via both its sensory and motor nerves to the muscular system, though the central connexions of these nerves are not yet completed.

This inactive period is followed by a period during which the muscular system is active, but the source of the stimuli arousing the central nervous system is not finally determined. The probable sources of these stimuli are discussed and the hypothesis is advanced that these movements represent the initial phase of reactivity in which changes in the external environment produce the activity via sensory nerves. Sensory collaterals at this stage have penetrated the alar lamina of the spinal cord.

This active stage passes into a reactive one during which direct stimulation of sensory fibres produces responses in the muscular system that vary in type with their central connexions and the degree to which the internuncial neurone system has developed.

Eventually the activity of the sensory fibres in several regions of the embryo simultaneously appears to be responsible for the segregation of muscle groups and a degree of inhibition so that localized stimuli tend to produce restricted muscular reactions.

A more general type of inhibition is exerted by regions of the brain lying ahead of the medulla oblongata. These "centres" in the brain that influence the activity

of the spinal mechanisms develop in a caudo-cranial direction. The first "centre" is in the mid-brain and by its influence on the spinal cord converts "jerky" movements into sustained ones. Still later "centres" develop in the mid-brain that convert the foetus into a modified decerebrate preparation. Following these "centres" are regions of the forebrain—not the cortex—that inhibit the activity of the whole foetus.

These "centres" acting on the spinal mechanisms differ during foetal life in their ability to tolerate asphyxiation. The lower "centres" are the most resistant; those that are most cranial are the least.

All of these processes appear to occur during the ontogeny of any mammalian embryo, but in some forms they appear to follow successively (sheep) whereas in others (rat) they occur almost simultaneously.

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PLANT TISSUE CULTURES

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I. INTRODUCTION

THE subject of tissue cultures in general, and of animal tissue cultures in particular, has been kept so constantly before the biological reading public during the last quarter century, by the work of Carrel, Fischer, Erdmann, Fell, Ephrussi, Lewis, and others, that it needs no introduction here. The subject of plant tissue cultures has received less public attention than has that of animal tissue cultures for the obvious reason that it has until very recently been much less successful. Haberlandt had clearly formulated the problem in 1902 (Haberlandt, 1902). Yet in 1931, twenty-nine years later, it was not possible to cite even a single example of a successful plant tissue culture (White, 1931). A review of that date (White, 1931) was quite properly closed with a quotation of Kuster's conclusion (1928) that the problem appeared to be "hopeless" (*aussichtslos*). Five years later it was still impossible to cite a single example of work which would fulfill the strict requirements of a true plant tissue culture (White, 1936*a*). But this time the problem no longer appeared hopeless, for two lines of attack, by Gautheret using cambium of trees (Gautheret, 1934, 1935) and by White using excised terminal meristems (White, 1932*a*, *b*, 1933*a*, *b*, *c*, *d*, 1934*a*), had begun to open up hints of the methods by which success might possibly be attained.

Since then the discipline has been rapidly acquiring momentum, and it is the purpose of the present review to bring the subject up to date by considering in some detail the information that has been amassed in the past four years and to outline briefly some of the probable lines of development for the future.

What is a "plant tissue culture"? In 1936 a "plant tissue culture" was tentatively defined as "any preparation of one or more isolated, somatic plant cells which grows and functions normally, *in vitro*, without giving rise to an entire plant"

(White, 1936*a*). This definition was purposely made rather broad and was left open to modification. It was pointed out that unlimited survival and growth should be made a part of any strict definition, but that the degree of differentiation permissible within the concept of a "tissue" culture was still an open question. La Rue (1936) would include the idea of totipotency, that is of capacity to develop into complete plants. While it is probable that all cells are to be looked upon as theoretically totipotent (White, 1939*e*), inclusion of this concept would make it almost impossible to distinguish between cuttings, which regenerate completely, and tissue cultures. This would create confusion. It seems better to retain a narrower definition and reserve three, or perhaps more than three, categories for the classification of cultures of plant materials. The three obvious categories are (1) tissue cultures, (2) organ cultures, and (3) cuttings. These are mere arbitrary divisions and any culture may conceivably develop into any one of these categories, depending on the conditions under which it has been grown. In the following pages the earlier definition will be somewhat curtailed. Henceforth, a plant tissue culture will be considered to be *any preparation of one or more isolated, somatic plant cells which grows and functions without serious physiological derangement yet without differentiating into distinct organs.* This corresponds to the original concept of Carrel (1924). By contrast, a plant organ culture is *any preparation of an isolated plant organ such as root, leaf, stamen, etc., which grows and functions without serious physiological derangement, without losing its original character and morphology.*

In reviewing the subject in 1936, a considerable part of the literature on regeneration and tissue repair in general was considered, not because these papers in any way dealt with tissue cultures as such, but because the methods developed in these studies and the information gained thereby represented such an important part of the background necessary before success with tissue cultures could be expected. This background need not be covered again. But, just as studies on regeneration led up to the first experiments on organ culture, which were discussed in this earlier review, it is precisely these organ cultures which have to a very considerable extent represented the background necessary for the successful consummation of tissue cultures. It is methods developed through the study of the former that have made possible the latter. And the latter have, in fact, only been successfully consummated within the last year and a half. I need offer no apologies, therefore, if I devote a considerable part of this article to a consideration, not of plant tissue cultures, but of organ cultures.

(1) *Organ cultures*

The cultivation of excised root and stem tips was originally proposed and a beginning made toward its consummation in the now classic studies of Kotte (1922*a*, *b*) and of Robbins (1922*a*, *b*) and Robbins & Maneval (1923, 1924). Chambers (1923), Malyschev (1932*a*, *b*) and Gautheret (1932, 1933*a*, *b*, *c*, 1935) likewise cultivated excised root tips and root cap cells. All of these cultures were, however, of relatively brief survival and did not establish the field as a working discipline. But in 1934, by slightly modifying the earlier methods, White showed that excised tomato roots could be grown indefinitely in an artificial nutrient consisting of a modified Uspenski solution, sucrose, and an extract of dried brewer's yeast (1934*a*). This immediately gave a great impetus to studies in the field and awakened a wide interest which has brought about many important developments. Other studies using this technique were undertaken in other laboratories. Gallagar studied the effects of types of seed-storage foods (starchy, oily, and proteinaceous varieties of corn and of other plants) on growth and development of excised seedling roots taken from the different types of seeds (1936, 1938, 1939). Gautheret found cystein hydrochloride to be beneficial to growth of excised roots (1935) and studied the behaviour of isolated root cap cells (1937*b*, *c*) and of fragments of embryos (1937*d*). Robbins, Bartley, and V. B. White studied the processes of regeneration of various types of excised root fragments (1936). Robbins, V. B. White, McClary & Bartley (1936) demonstrated the beneficial effect of certain unidentified ash constituents obtained from filter paper, agar, yeast and other organic materials on growth of excised corn and tomato roots. Some benefit was obtained by adding zinc, manganese and boron to the original nutrient, but this benefit was not equal to that obtained with ash of filter paper. Robbins & V. B. White (1936, 1937) also studied the effects of a number of different sugars, extracts of agar, pantothenic acid, and other materials on growth of excised corn roots. They failed to confirm Gautheret's conclusion as to the beneficial effect of cystein hydrochloride. They concluded that vitamin B_1 was of no benefit to corn roots, that "pantothenic acid" was possibly somewhat beneficial at 50 p.p.m., and that dextrose was the best sugar for use. Different brands of "pure" dextrose differed so greatly in effectiveness that the suspicion arose that this effect might be due to impurities in the sugars. They did not regularly obtain growth of corn roots free from abnormalities and, after review of the evidence, concluded that these abnormalities were probably due to some deficiency in the nutrient. Fiedler (1936) likewise failed to obtain unlimited growth of excised corn roots. His studies confirmed and enlarged the findings of earlier workers on the general requirements of excised corn roots. In addition, he found some suggestion that indole-acetic acid might stimulate growth of such roots, but that auxin rapidly disappeared from them after excision without their ceasing to grow at the same time. He concluded that auxin, while possibly beneficial, was not essential for growth and normal tropistic response of the roots. This represented a rather radical departure from the viewpoint of the auxin school and raised numerous objections from Boysen-Jensen (1937) and others, but has been partly supported by the findings of van Overbeek & Bonner (1938). Nagao, on the other hand (1936, 1937, 1938) found evidence of the production of auxin by excised root-tips of *Pisum sativum* and of *Zea mays*. Geiger-Huber (1936), Burlet (1936), and later Geiger-Huber & Burlet (1936) studied the effects of traces of indole-acetic acid and confirmed Fiedler's findings as to the stimulating effect of this substance at low concentration, but without following the subject farther.

Many of the studies cited above were already in progress before a publication by White in 1934 and, being made with roots which survived for only brief periods, are essentially continuations of the earlier work of Kotte and Robbins.

The nutrient or stimulating substances were chosen arbitrarily. With the demonstration that the excised roots of tomato could be grown for unlimited periods *in vitro* in an artificial nutrient, a set of completely adequate cultural conditions was established so that it was possible to turn from the earlier arbitrary *synthetic* method to an *analytical* one. The nutrient established at that time contained one constituent which, while it could be accurately duplicated at will, was, nevertheless, of unknown constitution. This was the 0·01 % of extract of dried brewer's yeast. This material, therefore, immediately became the object of intensive study and, while Gautheret, Fiedler, Geiger-Huber, Gallagar, Robbins, and others were seeking a satisfactory synthesis of a nutrient, analysis of the yeast extract was proceeding independently in the laboratories of Bonner, Robbins, and White. In December 1935, White reported (1935) that the effective material of yeast was completely soluble in water and 85 % alcohol, insoluble in ether, stable to boiling, destroyed by nitrous oxide, filterable, and diffusible through collodion, and that it consisted in major part of free amino-acids. It was apparently insoluble in 100 % alcohol. The importance of amino acids was suggested and it was pointed out that where these had been studied singly they were invariably depressant at concentrations above about 10 parts per million. The solubility in 85 % alcohol was confirmed in March 1937 by Robbins & Bartley (1937*b*) who, likewise working with tomato roots, reported evidence that the effective fraction could be partially, but not wholly, replaced by synthetic vitamin B_1. This was the reverse of their earlier finding with corn roots. Bonner (1937) had a month before (February 1937) come to the conclusion that vitamin B_1 was beneficial to growth of excised pea roots. In July 1937 White published papers (1937*b*, *c*, *d*, *e*) dealing in detail with this question. The necessity for yeast was established and the optimal concentration determined. The previously reported findings as to diffusibility, etc., were confirmed except for the apparent insolubility in 100 % alcohol. It was found that, if the material dissolved in 85 % alcohol was reduced to dryness and then extracted for 1½ hr. in three changes of boiling freshly distilled absolute alcohol, both the resultant extract and the residue, when dried and resuspended in nutrient solution, were without effect singly but gave normal growth when mixed (1937*c*). The fraction soluble in absolute alcohol was shown to be stable in hot acid but not in alkali, properties which suggested its affinity with vitamin B_1 (1937*e*) while the fraction insoluble in absolute alcohol had properties relating it to the amino acids (1937*d*). A mixture of nine amino-acids was developed which was capable of completely replacing this fraction when the nutrient contained sucrose, the standard salts of the modified Uspenski solution, accessory salts prepared according to the formula of Trelease & Trelease (1933), and the 100 % alcohol soluble yeast fraction. Vitamin B_1 had no effect if added to a nutrient containing only sucrose and standard salts, gave somewhat better results if the accessory salts were present and produced still better results if the amino-acid mixture was also present (1937*e*). With all of these materials present, however, the growth obtained was still somewhat inferior to that in a nutrient containing yeast extract. In September 1937, Bonner & Addicott published a somewhat similar analysis of the growth requirements for excised pea roots (1937). Their results agreed with White's findings on tomato roots as regards optimum temperature (*ca.* 27° C.), source of carbohydrate (sucrose), the necessity for yeast extract or its equivalent, and the beneficial effect of amino acids, and confirmed the findings of Bonner (1937), Robbins & Bartley (1937*a*) and White (1937*e*) as regards the essential nature of vitamin B_1. At about the same time, Robbins & Bartley (1937*c*) reported that, contrary to Bonner & Addicott's findings with pea roots (1937) and White's earlier findings (1934*a*), excised tomato roots would grow as well in solutions containing dextrose as in sucrose. In 1938 Robbins & Schmidt (Schmidt = Bartley) published an extensive recapitulation of the nutritional requirements of excised tomato roots (1938*a*). In addition to confirming a number of earlier findings, both of themselves and of others, they concluded that only the thiazole portion of vitamin B_1 was essential for this species. They found indole-acetic acid ineffective, a finding differing from that of Fiedler (1936), of Geiger-Huber (1936), and of Geiger-Huber & Burlet (1936) who worked on corn, but agreeing with that of Bonner & Addicott (1937) obtained in their studies on peas. They found cystein hydrochloride ineffective, in contrast to Gautheret's findings using corn and lupine (1935). In April 1938, White re-examined the accessory salt solution (1938*c*) used in previous work and by eliminating injurious elements arrived at a much simpler and more satisfactory solution capable of supporting approximately normal growth, in contradistinction to the relatively deficient growth reported by Robbins *et al.* in other "completely known" nutrients. This solution contained standard salts (1933*d*), salts of zinc, manganese, boron and iodine, sucrose, and the nine amino-acid mixture developed earlier. Bonner, in July 1938 (1938), and later Bonner & Buchman (1938), showed that excised pea roots required both thiazole and pyrimidine in equimolecular quantities for normal growth, these results differing from those of Robbins & Bartley (1937*b*) for tomato roots. In December 1938, Addicott & Bonner (1938) reported that nicotinic acid would satisfactorily replace the amino-acid mixture earlier used for pea roots and would, together with thiamin, give completely satisfactory growth. A month later, Robbins & Schmidt reported (1938*b*) that they could find no beneficial effect on growth of excised tomato roots from White's nine-amino acid mixture, but that vitamin B_6, identified as a probable constituent of certain impure sugars shown to be markedly beneficial, would support normal growth if added to the salts, sugar, and thiamin of the basic nutrient. This was expanded in a second paper in March 1939 (1939*a*), when it was shown that nicotinic acid and nicotinamide had no such effect on tomato roots as had been reported by Addicott & Bonner (1938) for peas. White had earlier reported (December 1938, 1938*e*) that the amino-acid mixture could be replaced by the single amino-acid, glycine, but not by any other of several single amino acids. White (1938*e*, 1939*b*) and Robbins & Schmidt (1939*a*, *b*) thus simultaneously arrived at two different, simple, completely known nutrients for the cultivation of excised tomato roots. Bonner & Devirian (1939) have recently brought some semblance of order out of this apparent chaos by showing that excised roots of pea and radish require vitamin B_1 and nicotinic acid, roots of flax require only vitamin B_1, while roots of their strain of tomato require vitamins B_1 and B_6. Evidence is also accumulating which indicates that within a given species two varieties may have quite diverse special requirements. It is thus unsafe to compare too closely results obtained in different laboratories.

These nutritional studies have been supplemented by a number of papers on other phases of the cultural requirements of roots. Loo & Loo (1935, 1936) concluded that extracts of leaves of a number of kinds of plants would stimulate growth of excised root-tips of the same or other species, so long as tannins were not present in the extracted leaves in quantities sufficient to precipitate the iron of the nutrient. Delarge (1938, 1939) studied the variation which occurs between roots of a single species grown in a given nutrient and concluded that stage of maturity of the seed and age of seedling or cutting from which roots were obtained had a marked effect on the rate and manner of growth, at least during the early passages after excision. Robbins, White, and Fiedler had all touched upon this question previously. White had studied the effects of a variety of nutrient solutions (1937*f*), concluding that the earlier modified Uspenski solution (1925) was somewhat superior to all others tested, largely because of its relatively low *p*H and low buffering capacity. He also studied the effects of various temperatures (1937*b*) on growth and survival. The weekly growth rates of a standard clone of tomato roots grown *in vitro* for three years, after correcting for the random variation noted by Delarge and others, showed a seasonal cyclic fluctuation in growth rate (White, 1937*a*). Variations in illumination proved to be negligible factors in this fluctuation (see also Robbins & Schmidt, 1938*a*), but such cultures are so sensitive to temperature changes that the cycle may possibly be entirely due to seasonal variations in temperature. White also showed (1938*d*) that roots of a considerable number of different species of dicotyledonous plants could be grown in the same manner and in the same nutrient, some of them, in fact, growing better than did tomato roots. Roots of some species did not grow satisfactorily under these conditions.

The study of excised plant organs, especially roots, has led then to a number of important conclusions as to their cultural requirements, some of which are clearly established, others still subject to disagreement. Excised roots of a number of dicotyledonous plants can be grown *in vitro* for potentially unlimited periods, in normal condition, on nutrients of known constitution. It is possible that roots of all such plants can ultimately be so grown. Those that have been grown do best at temperatures between 25 and 30° C. (White, Bonner & Addicott). They are little, if at all, affected by moderate illumination (White, Robbins & Bartley). They require most, if not all, of the salts included in the classic nutrient of Pfeffer (Bonner, Robbins *et al.*, White). Ferric sulphate appears to be a better source of iron than are other iron salts such as ferric chloride (White, Robbins & Schmidt), but Robbins and Schmidt believe this superiority to be due to impurities in this salt rather than to any difference in availability (1938*a*). They require certain accessory salts such as manganese, zinc, and boron (Robbins *et al.*, Bonner & Addicott, White), and according to White also iodine (1938*c*). They require some sugar, but, while White found sucrose essential and Bonner & Addicott and Fiedler found it to be superior to dextrose, Robbins & Schmidt found both sugars, as well as some others, to be of about equal effectiveness. They require vitamin B_1 or its precursors (Bonner, Robbins & Schmidt, White). They also require some additional accessory organic material. Addicott & Bonner found nicotinic acid an adequate source of this material for pea roots (1938), and White found a nine-amino acid mixture (1937*d*) or simply glycine (1939*d*) to serve the purpose for tomato roots, while Robbins & Schmidt (1938*b*; 1939*a*, *b*) and Bonner & Devirian (1939) reject both the amino acids and nicotinic acid for tomato roots and claim that vitamin B_6 is the essential factor. The real position of these materials is far from settled. The position of indole-acetic acid and cystein hydrochloride is likewise still in doubt. But the major problems in establishing nutrients of known constitution for the cultivation of excised plant roots have been solved.

(2) *Tissue cultures*

The numerous studies of isolated organs, especially roots, outlined above have given us considerable insight into the conditions which plant cells require for satisfactory growth. They represent a laboriously built up foundation without which successful cultivation of isolated tissues cannot be expected. The cement of this foundation is hardly yet dry and it is not surprising that little progress has yet been made on the building itself. It is true that some promising work had been done long ago. Gautheret, in 1934 (1934, 1935) when the cultivation of excised roots was still in its infancy, had already shown that cambium of *Salix capraea*, *Populus nigra* and other trees could be made to grow for some months *in vitro*, forming very interesting algoid outgrowths, undergoing cell division, etc. The nutrient used contained only the standard salts of Knop solution, glucose, and cystein hydrochloride. Such cultures did not survive indefinitely but did show much promise. Bonner cultivated ovarian parenchyma of *Phaseolus vulgaris* through several passages, the experiments always ending, however, in cessation of growth, and death (1936). Gioelli (1937*a*, *b*, 1938), using the same solution as had Gautheret, had obtained some growth of cambium of *Sterculia platanifolia*, *Ailanthus glandulosa*, and *Ulmus campestris*, but again without indefinite survival and growth. It is only with the introduction of thiamin into the nutrients and with the realization of the importance of the accessory salts, both developments resulting from studies on excised roots, that real success with undifferentiated tissues, such as cambium, has been attained. By adding thiamin and indole-acetic acid to his nutrient, Gautheret in October 1937, succeeded in greatly stimulating the growth of cambium of *Salix capraea* (1937*e*, 1938*a*). Nobécourt, a month before (1937, 1938*a*, *b*) had reported the growth of undifferentiated root tissue of carrot and potato for two months in a similar nutrient to which accessory salts were also added. Nobécourt subsequently reported obtaining continued growth of carrot tissue for a period exceeding 20 months, through numerous passages. The details given in his papers are too meagre to permit a real opinion of their importance (1939*a*, *b*, *c*). Gautheret, combining his own and Nobécourt's techniques, studied the behaviour of excised carrot and kohl-rabi tissue in culture (1938*b*, *c*, *d*, 1939*c*). But it is only with the last weeks of 1938 and the beginning of 1939 that full and indubitable success in this direction has been attained. This goal has been reached independently and almost simultaneously, but with quite different materials, by Gautheret (1939*a*, *b*), by White (1938*e*, 1939*a*), and perhaps by Nobécourt (1938*a*, *b*).

On 9 January 1939, Gautheret presented before the French Academy of Sciences (1939*a*) the results of studies constituting a combination of his own previous work with that of Nobécourt. Using a Knop solution supplemented with Berthelot's mixture of accessory salts (1934), glucose, gelatine, thiamin, cystein hydrochloride and indole-acetic acid, the last constituent at much lower concentration than had Nobécourt (1937), he had cultivated fragments of carrot through seven passages of about two months' duration each. These showed little or no differentiation beyond the formation of occasional lignified cells in the interior of old fragments. They grew slowly and without any indication of diminution in growth rate. The record, though brief, if taken with Gautheret's earlier work, is sufficient to justify the conclusion that he has obtained cultures satisfying both major criteria of a plant tissue culture—potentially unlimited growth, and undifferentiated growth—so that there need be no further doubt as to the real success of his efforts. Gautheret laid the necessary foundation of technique, Bonner, Robbins and White introduced the use of thiamin, Nobécourt contributed the idea of using carrot tissues and the accessory salts. The accessory salts had been independently introduced by White and Robbins, and Gautheret and Nobécourt simultaneously obtained apparently satisfactory results.

White's paper on cultivation of undifferentiated callus tissues was presented before the meetings of the American Botanical Society on 30 December 1938, ten days before Gautheret's (1938*e*). Using the same nutrient as that developed for excised roots, containing the standard salts, sucrose and yeast extract, stiffened by adding 0·5 % of agar, and employing procambial tissue taken from young stems of a hybrid *Nicotiana* (*N. glauca* × *N. langsdorffii*), cultures were maintained through forty passages of one week's duration each. During this time they increased about threefold each week resulting in a theoretical increase of 3^{40} times. The structure of the cultures was very similar to that noted by Gautheret & Nobécourt. There was no evidence of differentiation beyond the formation of an occasional scalariform cell in the interior of the tissue masses. These cultures are now (June 1939) about the same age as those reported earlier by Nobécourt & Gautheret (19 months) but, having been divided and subcultured each week, they have been through about eighty passages as against Nobécourt's eight passages and Gautheret's seven. There seems to be no reason to doubt that these cultures do satisfy both criteria of tissue cultures, and, with those of Gautheret & Nobécourt, represent the first real tissue cultures of plant material.

As was said in the introduction of this paper, the division of cultures of plant material into tissue cultures, organ cultures and cuttings is an arbitrary one and conditions of cultivation may greatly modify the behaviour of any given tissue. This is especially brought out by the callus cultures. If maintained on the standard 0·5 % agar nutrient mentioned above, they behave as described. But if they are placed in a nutrient lacking the agar, they differentiate, forming stems and leaves (1939*b*). They thus become no longer tissue cultures but cuttings similar to those described by La Rue (1936) and Behre (1929). The transition from category 1 (see above) to category 3 is abrupt and seems to be determined by a single variant in the environment, presumably oxygen supply (1939*e*).

II. APPLICATIONS

True plant tissue cultures are of such recent occurrence that there has as yet been no time to apply them to extraneous problems although Riker & Berge (1935) and others have discussed their possibilities. Nobécourt, indeed, was led to attempt such studies because of his interest in crown-gall tumours of plants (Nobécourt & Dusseau, 1938). Only one study of the behaviour of plant tissue cultures has been made to date, that of Plantefol (1938), who studied the respiration of some of Gautheret's cultures of cambium from *Salix capraea*. But plant organ cultures have been in existence long enough to find certain applications in a variety of fields. One of the earliest was in the maintenance and study of plant disease viruses. By obtaining roots for culturing from tomato plants systematically infected with plant virus diseases, *in vitro* cultures carrying a number of such viruses in diseased roots have been obtained and maintained for about five years (White, 1934*b*). Using these cultures it has been possible to show that, although one of the most marked symptoms of disease in intact plants is commonly the alteration in chlorophyll behaviour, this is in fact a secondary symptom, for the viruses will multiply quite as well in the complete absence of chlorophyll (White, 1934*b*). The rate of multiplication of the viruses appears to be independent of the rate of multiplication of host cells (White, 1936*b*). The chemical and physical characteristics of virus proteins isolated from such cultures is indistinguishable from those of proteins isolated from green tissues of intact plants, although the concentration present in cultures is relatively small (Stanley, 1938). Lewis & McCoy had already in 1933 (1933) used a somewhat similar method in studying the formation of nodules on legume roots by nitrifying bacteria. From these few examples the possibilities of the method in the studies of problems in plant pathology will be evident.

Root cultures likewise provide excellent materials for work on plant tissue nutrition as has already been made evident from the studies on the function of thiamin and of the carbohydrates. They are equally useful in studies of inorganic nutrition. Nowhere is this made clearer than in the case of iron. As with the viruses, the most evident symptom of iron deficiency in intact plants is the chlorosis produced, and one might easily conclude that the failure of such chlorotic plants to grow was a matter of carbohydrate starvation due to failure of the photosynthetic apparatus. But root cultures deprived of iron, although well supplied with carbohydrate and possessing no apparatus capable of chlorotic change, nevertheless show an immediate and profound failure. Growth stops within 24 hr. after transfer into a nutrient lacking iron. Here the failure is evidence, not of secondary starvation, but of primary injury, presumably through failure of the respiratory apparatus. The respiratory function of iron is brought into sharp relief and justifies the hope that studies such as that of Plantefol (1938) already mentioned may lead to important results.

Root cultures have also been used in studying tissue secretion as exemplified in the phenomenon of "root-pressure" (White, 1937*g*, 1938*a*, *b*). Roots have been shown to secrete water basipetally, in a rhythmic fashion, under pressures exceeding 6 atmospheres. The results suggest that enormous forces emanating from the living cells themselves may well be involved in the movement of fluids through plant tissues, and, since the most obvious source of energy for the development of such forces seems to be energy of respiration, these studies tend to emphasize the importance of a careful analysis of the respiration processes going on in such materials.

III. DISCUSSION AND OUTLOOK

If one compares the record of the last four years as set forth in these pages and in the reviews of Fiedler (1938), Gautheret (1937*a*, 1938*e*), Nobécourt (1939*c*) and White (1939*b*, *c*) with that given in the earlier reviews of 1931 and 1936, the almost explosive development of the subject will be quite evident. As is so often true in science, this field has been opened up simultaneously from several directions. Much progress has certainly been made. Nevertheless, the original aim of tissue cultures as set forth by Haberlandt nearly four decades ago was the cultivation of single cells. And this has probably not yet been accomplished. Schmucker, it is true, reported success in this direction a decade ago (1929), but his work has never been confirmed. This still remains a challenge to workers in the field and will doubtless receive much attention in the future. The milestones in the history of plant tissue cultures so far have been: (1) formulation of the idea in 1902 (Haberlandt), (2) cultivation of excised roots *in vitro* for limited periods in 1922 (Kotte, Robbins), (3) cultivation of excised tomato roots for potentially unlimited periods in an artificial nutrient in 1934 (White), (4) demonstration of the significance of thiamin for growth of isolated plant tissues in 1937 (Bonner, Robbins & Schmidt, White) and (5) cultivation of undifferentiated plant callus *in vitro* for unlimited periods in 1939 (White, Gautheret, Nobécourt). The cultivation of single cells remains a major unsolved problem. Doubtless much effort will be expended in perfecting the present methods, extending them to other species, and applying their use to practical problems. As yet no monocotyledonous root has been successfully grown for long periods, in spite of much work directed at that object. Fiedler believes this to be due to a fundamental difference between seedling roots and adventive roots (1936), but since all the virus-bearing root cultures maintained in this laboratory have been derived from adventive roots this does not seem at all probable. It is more likely that the defect lies somewhere in the techniques used. This problem should be studied more carefully. Many questions in regard to nutrition of tissues remain to be solved in spite of the fact that such tissues can be grown in nutrients of precisely known constitution. The functions of the different elements and the detailed effects of deficiencies of one or more of them is still practically untouched in root-culture studies except for brief references in the work of Robbins *et al.* and of White, yet the study of, for example, iron deficiency in a chlorophyll-free organ or tissue growing *in vitro* certainly offers great possibilities. The question of tropistic responses of excised roots has been touched upon by Kotte, Robbins & Maneval, Fiedler and others, but remains to be studied in detail. The respiration of excised roots has been untouched, although Plantefol has made a brief study of respiration of growing excised callus (1938). The recent discovery that excised roots maintain a rhythmic basipetal flow of liquid, extruded against huge pressures, opens up a number of interesting possibilities with regard to secretion and other functions of simple tissues.

The pathology of isolated tissues has been touched upon by White, Lewis & McCoy, Gioelli and others, but is a relatively virgin field. The anatomy and morphogenesis of such cultures has likewise been barely touched.

These suggestions are set forth merely to show some of the many possibilities opened up by this relatively new method of studying life processes. In the last few years the method of plant tissue cultures has at last taken its place in the armamentarium of the plant physiologist and plant pathologist. The next few years should find its importance and usefulness greatly increased.

IV. SUMMARY

The problem of cultivation of excised plant tissues, originally formulated in the early years of this century in an effort to study the interrelations of cells considered as "elementary organisms" (Haberlandt, 1902), remained, in spite of intensive investigation and in spite of some promising leads such as those of Kotte & Robbins (1922), essentially unsolved until 1934. Since then great progress has been made and most of the fundamental questions of technique have been solved.

Excised roots of many species of plants have been cultivated *in vitro* as isolated units for long periods and can safely be considered potentially immortal under the conditions used. Their nutrition has been studied in considerable detail. They require an external source of sugar, and of organic nitrogen in the form of glycine or some other comparable material. They are extremely sensitive to deficiency of thiamin (vitamin B_1) which must also be supplied in the nutrient. They have rather rigid requirements as regards some of the mineral salts, especially iron. Their requirements as regards some other salts are less rigid. Other phases of their physiology, such as their capacity to secrete water against osmotic and hydrostatic pressure gradients, have been studied briefly. Their pathology with respect to certain plant virus diseases has also been examined.

Cultures of various types of undifferentiated tissues have been made, mostly within the last two years. The most successful of these have been the procambium cultures of White, the cambium cultures of Gautheret and the carrot root cultures of Gautheret & Nobécourt. The influence of oxygen tension in determining differentiation has been studied in the first of these.

The present review considers in some detail the papers that have appeared between January 1936, and January 1940, and outlines briefly the probable future trends in this field.

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GROWTH-PROMOTING NUTRILITES FOR YEASTS

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I. GENERAL AND HISTORICAL INTRODUCTION

(1) IMPORTANCE OF YEAST STUDIES

SOME of the most far-reaching discoveries in the whole field of biology have been made in connexion with the study of yeasts. The fruitful doctrine of the microbial origin of human and animal disease had as its forerunner the studies of Pasteur on the "diseases of wine" which could be "cured" by avoiding microbial contamination of the yeast cultures. Likewise the familiar doctrine that disease may be caused by the lack of vitamins had its forerunner ten years before Funk's promulgation of the "vitamin hypothesis" in the studies in Ide's laboratory in Louvain (Wildiers, 1901), where it was discovered that yeast required for its proper nutrition traces of a mysterious substance (or substances) which was called "bios". Whatever basis there is for the statement that the first vitamin was discovered at this time (1901) will be apparent from the facts recorded later in this review. The insight which we now possess regarding the fundamental process of carbohydrate utilization is based in a large measure on studies of alcoholic fermentation by yeast and the enzymes from yeast. Even the word *enzyme* means "in yeast".

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[1] I wish to express my thanks to my colleague, Dr E. E. Snell, for valuable suggestions in connexion with the preparation of this review.

(2) EARLY HISTORY OF "BIOS"

Since the earlier history of "bios" has been reviewed previously (Tanner, 1925; Miller, 1930; Peskett, 1933[1]),[2] I will omit, for the most part, a discussion of the earlier literature, and will limit myself to a brief sketch which I hope will bring out points not hitherto made clear.

Pasteur had observed that if yeast, the size of a pin head, were introduced into a solution of sugar, ammonium tartrate and the minerals in yeast ash, propagation and fermentation resulted. Wildiers (1901), working under Ide's supervision (R.J.W.[3] 1938), reported that yeast failed to grow appreciably in such a solution from a very small seeding, but that propagation was induced when a small amount of a sterile decoction of yeast, for example, was introduced into the medium. The results obtained were clear-cut and the interpretation was sane. While the hypothetical substance was called "bios", it was recognized even at that early date that more than one substance might be responsible for the observed growth stimulation, but the name was coined to designate whatever agent or agents might be responsible for the effect. It was proved not to be present in the "ash" from extracts. Experimentally the growth of yeast was judged qualitatively by observation and quantitatively by noting the loss of weight (carbon dioxide) resulting from fermentation. Observations were continued for many days, and when no visible (or weighable) fermentation took place in the solution containing only sugars and salts (observed in some cases for 15 days) the investigators felt justified in concluding that the "bios" was *indispensable* for the growth of yeast.

The question of indispensability was the basis for considerable unproductive dispute and even quibbling, while the active chemical principle (or principles) contained in the "bios" solution remained undiscovered. No one carrying out experiments similar to those of Wildiers could have failed to recognize the presence of *something* in a yeast extract highly stimulatory to yeast growth. It was easier to deny the indispensability of that something than to find out its chemical nature.

The fact that sucrose, ammonium tartrate and asparagin, all derived from plant sources, were frequently presumed to be pure substances when actually they contained some "bios", may have led to a failure to recognize the "bios" effect. Even "lecithin" and "peptone" were thought of as pure by some investigators. Another fact which may be offered in extenuation of the error of the workers who denied the existence of "bios" is that different strains of yeast differ greatly in their ability to grow in a medium of simple composition, and the strains with which the work in Ide's laboratory was done were probably more dependent upon "bios" than many strains.

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[1] *Biological Reviews.*

[2] For other articles containing related review material see Pulkki (1934), Dagys (1935), Kogl and Tonnis (1936), Kogl (1937), Koser & Saunders (1938), Lwoff (1938), Thimann & Bonner (1938), Fulmer (1939).

[3] References to R. J. Williams are cited as R.J.W.

Certain earlier workers postulated that "bios" was not an essential, but was effective in a yeast culture only because it counteracted the effect of copper or other poisons which in its absence prevented growth. Other writers took the position embodied in the quotation from Tanner (1925): "yeasts developed better in a medium to which yeast water had been added. Whether the stimulation in growth was due to 'bios' or to other materials in the extract was not known." Miller (1930) spoke of those who took such a position as "qualified to join the Last Ditch Bacon Club, which holds that Shakespeare's plays were written not by Shakespeare, but by some other bearing the same name".

Considering the enormous literature regarding yeast which exists, especially that connected with the brewing industry, the original publication on "bios" made no more than a ripple. The more influential writers tended to deny the indispensability of anything such as Wildiers described and between the years 1906 and 1919 "bios" almost completely dropped out of the chemical literature. At the later date there were many qualified experts in the fermentation industries who had never heard the name.

(3) POSSIBLE RELATION TO VITAMINS; NUTRILITES

In 1919 at least three articles appeared bearing directly on the subject. The first to be submitted was my own doctorate dissertation (R.J.W. 1919). This investigation was carried out under the supervision of Prof. F. C. Koch at the University of Chicago and was supported by the Fleischmann Company. Yeast ("old process" strain, Fleischmann) was observed microscopically growing from single cells in hanging droplets of media of different compositions, and was found to require for extensive multiplication an unknown organic constituent (or constituents) of wort or yeast extracts. The earlier investigation on "bios" was referred to and discussed in connexion with these results.

It was shown (R.J.W. 1919) "that the substance promoting the growth of yeast occurs in the same materials as those in which vitamine has been found; namely protein-free milk, wheat germ, lactose (Kahlbaum), yeast, egg yolk and pancreatin. The substance is none of the commoner amino acids contained in an acid digest of casein with tryptophane added. It has the same properties of solubility, precipitation by phosphotungstic acid, adsorption on fuller's earth, heat stability, and behaviour toward acid and alkali, as nearly as we know these properties, as the water soluble vitamine and in addition the two substances so far as known have no divergent properties....From the cumulative evidence offered we believe we are justified in concluding that as far as present knowledge is concerned the substance or substances which stimulate the growth of yeast is or are identical with the substance or substances which in animal nutrition prevent beri-beri or polyneuritis."

It should be remarked that at this time the term "water-soluble vitamine" was supposed to designate a single vitamin possessing the antineuritic and other properties now ascribed to the "vitamin B complex". The existence of two water soluble "B" vitamins was first seriously suggested (Mitchell, 1919) shortly after the publication referred to above.

This suggestion of a vitamin affecting the growth of yeasts was anticipated by Kita (1914) who observed, as had Kluyver (1913) before, that crude maltose is more easily assimilated by yeast than a highly purified product, and suggested that an "oryzanin-like" substance was responsible. Kurono (1915), another Japanese worker, more definitely ascribed the stimulative effects of rice bran and yeast extracts to "oryzanin". Though he stated that oryzanin had been obtained in pure state the year before by Suzuki (a claim which was not, however, substantiated), he did not carry out tests with anything but crude materials. The two articles by Japanese workers were published in obscure journals and hence did not attract the attention which they merited.

Very shortly after my first article was submitted, one was presented by Abderhalden & Koehler (1919) in which it was shown that the growth of yeast and certain other organisms was accelerated by yeast extract. However, no mention of "bios" or "vitamine" was made. Within a few weeks Bachmann (1919) also published evidence indicating that yeast fermentation was stimulated by vitamin-like material. She concluded: "The amount of organic material required is so small and the effect upon the growth and activity of the yeast is so striking that a marked similarity exists between these organic substances and the vitamines required for the normal development of animals."

The possible relationship of vitamins to yeast growth greatly stimulated interest in "bios" and as a result a large volume of work has been published in the last twenty years. However, comparatively few laboratories undertook serious studies to isolate and identify the active substance or substances responsible for "bios" activity. An unfortunate amount of energy was spent (1) on the question of "indispensability", (2) in formulating modified media (not containing "bios") which might be slightly superior to other basal media, and (3) in attempting to decide on the basis of crude materials the identity or non-identity of "bios" and "vitamin B".

It is now well known, of course, that the whole "bios" effect cannot be ascribed to any single substance, that "vitamin B" activity is due to a considerable number of separable organic compounds, and that there is much overlapping so far as the responsible agents are concerned. Our present state of knowledge would have been reached earlier if attempts at chemical isolation had been stressed from the first.

Especially in recent years the term "vitamin" has been used almost universally to designate organic substances which in minute amounts are of importance in *animal* nutrition. Because of this fact the term *nutrilite* was coined by myself (R.J.W. 1928) to include organic substances which in minute amounts function in a similar manner for other types of organisms. The term vitamin carries unfortunate etymological implications and its use should not be unduly extended. The term nutrilite, on the other hand, is unobjectionable from this standpoint, since it implies only an importance in nutrition and nothing as to the chemical nature of the agent.

(4) CAUSES FOR CONFUSION

Nearly forty years have passed since "bios" was discovered, and still the chemical agents involved have not all been identified. During this time there has been an extraordinary amount of confusion and distrust on the part of "outsiders" who could hardly form an intelligent opinion on the subject of yeast growth-substances because of the conflicting reports and opinions. Before entering into the main discussion of this review pertaining to specific yeast nutrilites, it seems desirable to point out some of the fundamental reasons for the earlier discordant results and interpretations. The reader can then better appreciate the present status of the subject, in which the confusion (but not the complexity) has largely disappeared.

In discussing this confusion I have no desire to allocate the "blame" for it or the "credit" for the constructive steps which have resulted in its partial disappearance. All of those who have experimented have to some degree helped in dispelling the fog, and no word of criticism or apology should be directed toward those who elected to do their experiments in their own way rather than to fall into the patterns set by previous investigators.

(*a*) *Differences in "strains" of yeast*

The foremost cause of confusion probably dates back to the dispute between Liebig and Pasteur and is due to the striking differences in behaviour between "strains" of yeast which are practically indistinguishable morphologically. W. L. Miller (1930) says in this connexion: "If it had only been recognized at the time that different kinds of yeast behave quite differently in this respect, the quarrel between Liebig and Pasteur which poisoned the literature of this subject for nearly fifty years need never have occurred." The same might be said of numerous other differences of opinion with regard to "yeast". We are not concerned here with differences which exist between entirely different types of yeasts such as have been considered by Tanner *et al.* (1926), Copping (1929), Farrell (1935) and Elder (1936). We are referring to "strain differences" within what has been considered one species, i.e. *Saccharomyces cerevisiae*.

It is difficult for one who has not experimented on the nutritional requirements of yeasts to appreciate how diametrically opposite the results may be when one investigator works with one strain and another chooses to do his experiments with another strain of *S. cerevisiae*. In my laboratory different strains have been cultured for more than 15 years without changing their characteristics. Possibly the most striking case of disparity of behaviour is that of "old process" yeast (also Yeast no. 578 of the American Type Culture Collection) which is *highly* responsive to vitamin B_1 when the proper supplements are supplied, but in this respect differs markedly from several other strains.

Different strains show marked differences in their synthetic ability and also in the ease with which they adapt themselves to a new medium. In certain experiments "old process" yeast, for example, appeared to have very weak synthetic powers until it had been in the medium for about 36 hr., after which it seemed to develop marked synthetic ability which enabled it to grow steadily thereafter in a simple medium.

Certain yeasts which require a given nutrilite for rapid growth appear to respond to different concentrations to such an extent that a "maximum" dose for one yeast is hardly enough to give a measurable response with another. Such behaviour may result in two observers obtaining positive and negative results, respectively, even when the fundamental requirements in the two cases are the same (which is, of course, not always the case).

(*b*) *Diversity of experimental procedures*

Another important cause for the differences in the results of different investigators is the fact that there are so many different ways in which yeast growth experiments can be performed.

Whether a heavy, light or extremely light seeding of yeast is used may determine the character of the results to a large degree. When one seeds with heavy suspension, there is not room in the medium nor food material enough for extensive multiplication. As a result the requirements of the yeast under these conditions may be entirely different from those which would appear if multiplication were extensive. Kögl and co-workers, in their investigation of "biotin", seeded the medium heavily and obtained (under test conditions) up to about 120% increase over the initial seeding. Under these conditions the yeast responds to biotin markedly, but does not require, for example, an outside source of pantothenic acid, evidently because there is enough of this substance present in the mother cells to allow propagation without depletion of one new generation.

The time interval of growth may also determine the character of the results. If on the one hand a yeast culture is allowed to grow only a short time after acclimatization, essentially the logarithmic growth rate may be measured, whereas if it is allowed to grow to the extreme limit something quite different is involved. Different media may give results which are in no wise parallel when tested in these two different ways.

Because of the difficulties of control, aeration, though theoretically desirable, has not been used extensively in experimental studies. It was shown long ago by Pasteur that aeration greatly increases a yeast crop.

The Toronto workers (Clark, 1922; Miller *et al.* 1933) have made some approach to ideal conditions by growing the yeast in rocker tubes and have shown that in vessels of different sizes or shapes the yeast crops under static conditions are not the same because of differences in settling. In my laboratory parallel experiments have been carried out both with and without agitation (unpublished results) and whereas, as was anticipated, the yeast crops were different in the two cases, we were in general able to observe the same phenomena and the same superiority of one medium over another following either procedure. The procedure which does not involve shaking is theoretically undesirable, but practically it is a method which lends itself readily to obtaining significant results from numerous simultaneous tests.

Tanner, in his review (1925), criticized those who did not use strict bacteriological technique in the application of tests and stressed the probability that contamination entered to vitiate the results. In my experience this has not been a common source of error. In the first place yeast grows in a relatively strongly acid medium which is unsuitable for bacteria. This fact finds important application in the yeast industry and makes the manufacture of yeast vastly simpler than would otherwise be the case. So far as I am aware, those who have not used strict bacteriological technique throughout have depended on short growth periods. In such cases if a medium is seeded, for example, with 60,000 cells per ml., from a pure culture (still, however, constituting a light seeding), the yeast crop after 16 or 18 hr. will be practically entirely the progeny of the seed yeast even though the medium was sterilized only by steaming or even by pasteurization.[1] No one would deny that such cultures would ultimately become significantly contaminated, but experience has shown that in a short growth period they do not. It was found in my laboratory that whether flasks in which such cultures are grown (16–18 hr.) are plugged with cotton or not the results are the same, hence literally tens of thousands of test cultures have been grown in my laboratory in which the flasks, glassware and media were sterilized only by steaming, the seeding (originating from a pure culture) done in the open and the flasks left exposed to the air during the entire incubation period. When long incubation periods are used it is of course necessary to use the most careful technique. The sterilization must then be especially efficient to avoid mould contamination.

Numerous ways have been used for evaluating yeast crops. These include counting the cells directly in hanging drops (R.J.W. 1919), using a hematocytometer for counting (Souza & McCollum, 1920; Clark, 1922), weighing the yeast (R.J.W. 1920), centrifuging in calibrated tubes (Carlson, 1913), visual comparison of turbidities (Peskett, 1927*a*), use of a special thermocouple turbidimeter (R.J.W. *et al.* 1929) and use of a photoelectric turbidimeter. "Bios" effects are large enough so that qualitatively or even semi-quantitatively any of the methods mentioned above yield significant results. They have all been used in my laboratory. Only the last two are satisfactory for highly quantitative work and of these the thermocouple apparatus is definitely superior. No serious confusion has been caused because of the use of different methods in different laboratories.

Divergent results have been obtained in different laboratories because of the use of different culture media. A medium which is suited to one yeast may not be entirely suited to another, and results obtained when two different basal media are used will not always agree. In my laboratory, for example, the effect of the presence of variable concentrations of asparagin (or aspartic acid) on the character of the results has been shown (R.J.W. *et al.* 1939). The recent studies of Fulmer and co-workers (1936) show how the concentration of magnesium sulphate in the basal medium may be a determining factor.

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[1] Significant results can also be obtained in short-period experiments by somewhat heavier seeding from impure cultures (compressed yeast), though this involves complications (R.J.W. 1920). This procedure is not now recommended partly because, from time to time, yeast manufacturers change the strains which they propagate.

(*c*) *Difference in source of nutrilites*

Since yeast growth stimulation in varying degrees may be brought about by the presence of single purified nutrilites or by various mixtures of these active substances, it follows that nutrilite-containing extracts from different sources should not be expected to yield similar results when subjected to treatments or to fractionation procedures, since their physiological effects may be based upon the presence of entirely different proportions of the different nutrilites. In my laboratory sufficiently different dosage-yeast-crop curves have been obtained with extracts from different sources (e.g. rice bran, tomato and liver) to make it appear certain that yeast growth stimulation at different levels of dosage was predominantly influenced by different nutrilite constituents. On the basis of these results I could feel no assurance, for example, that a "Bios IIB" preparation obtained from two sources by adsorption would owe its physiological activity solely or even predominantly to the presence of the same specific nutrilite. This is partially due to the lack of specificity of charcoal adsorption.

I feel, however, that tolerance for other investigators' methods is desirable. I am aware that many workers in the field reacted unfavourably to the "biotin" investigations of Kögl and co-workers, because unlike previous work, large seedings of yeast were involved. This seemed unjustified on theoretical grounds, but actually led to the discovery and isolation of one of the most interesting compounds among the yeast nutrilites. If small seedings had been used (and multiplication had therefore been extensive), the discovery of "biotin" would probably have had to await the isolation of pantothenic acid and other nutrilites because, according to experience gained in my laboratory, a test for biotin involving extensive multiplication must, in order to be specific, utilize the presence of other purified nutrilites in the basal medium (R.J.W., Eakin *et al.* 1940).

(*d*) *Unrecognized potency of pure nutrilites*

It appears that premature claims sometimes have the effect of stimulating interest and research, but this is not always the case. The very early claim of Devloo (1906) that he had isolated "bios" in the form of lecithin certainly caused confusion and probably tended to discredit the whole study and was a factor in causing the abandonment of "bios" studies for a number of years.

I myself lay no claims to exemption, so far as making premature claims is concerned, and am aware that I was over-optimistic when in 1919 I accepted so definitely the idea that yeast required for its nutrition the same vitamin which was required by animals. The progress of the study would probably have been forwarded if I had been more sceptical and less definite in my conclusions.

In the earlier years investigators had no basis for realizing how exceedingly potent the pure chemical principles responsible for "bios" activity might be. Thus when Eddy and co-workers (1924) announced the isolation of a substance with "bios" properties which was effective in concentrations of 0·005 mg. per ml., the high potency of pantothenic acid, for example, could not have been anticipated. The latter can be quantitatively determined under conditions similar to those used by Eddy and co-workers when 0·0000008 mg. per ml. is present and in some tests 0·00002 mg. per ml. gives about the maximum response. Biotin, under somewhat different conditions, is detectable in still smaller doses as will be discussed in a later section. The "bios" isolated in 1924 could have been more than 99·9% *im*pure and still have caused the response attributed to it.

Certain workers failed to realize how striking "bios" effects are, and thus attributed them to the presence of inorganic constituents in spite of the repeated statements that the "ash" of extracts do not contain the active principle. One such study led to the bona fide observation that asparagin contains a trace of thallium (Richards, 1932) and that this trace may cause some yeast growth stimulation. The "bios" effects, however, as they are manifest, for example, in a culture grown 24 hr. from a small seeding do not involve a mere 25, 50 or 100% increase over the blank, but when sufficient doses are used, cause yeast crops more than one hundred times as large as when no addendum is made.

Now that the extreme potency of purified "bios" constituents is established, investigators should be on their guard against thinking of crude preparations as though they represent single substances. A sharp distinction should be made (but this is not always done) between an extract which contains some biologically active principle and a preparation which contains a single biologically active substance. A preparation, in order to be recognized as of the latter type, must at least be physiologically active in minute doses as judged by present standards.

Because the field of "bios" investigations has now been developed to the point where recognized chemical substances have been discovered to play leading roles, the main body of this review will be written from the standpoint of these specific substances. They have been shown to be effective for various strains of *Saccharomyces cerevisiae* which are used in the baking and brewing industries. We shall not attempt to mention all of the numerous studies which have been concerned with yeast growth stimulation, but only those which have to do with purified nutrilites or which have led to their purification.

II. SPECIFIC YEAST NUTRILITES

(1) Inositol

The first pure substance found to contribute definitely to "bios" activity was inositol. When Eastcott (1928), from W. L. Miller's laboratory, first announced her findings, extended and conclusive evidence was presented to show that the substance which she had isolated from tea was actually inositol. The evidence, however, indicating that the pure substance had "bios" activity was not presented in detail and it appeared plausible that the physiological activity possessed by her preparation might be due to impurity and that other inositol preparations (e.g. Kahlbaum inositol) obtained from plant sources, might owe their physiological effects to traces of highly potent impurities. The plausibility of this assumption was enhanced by the fact that inositol was always used in the original experiments in relatively high concentrations ($\frac{1}{60}-\frac{1}{3}$ mg. per 10 ml.), and by the fact that Kahlbaum's lactose had been found previously to contain "vitamine" (McCollum & Davis, 1915) and also "bios" (R.J.W. 1919).

Subsequent investigations, however, have proved that such assumptions were in error and that physiological activity resides specifically in the inositol itself and other closely related compounds cannot replace it.

One of the striking facts regarding inositol (aside from the relatively high concentrations that are required) is that when added by itself to a synthetic medium, the effect is practically nil. It becomes a limiting factor for growth only when other nutrilites are supplied, and the failure of earlier investigators to confirm its effect was due to lack of the proper supplements. It has been shown to have some effect on all of the strains which have been adequately tested. It is effective in the high seeding tests of Kögl as well as the low seeding tests of other workers.

In her original report Eastcott grew a strain of yeast without inositol and determined by the physiological response to the extract of such yeast that it contained about one-half the normal amount of inositol. The experimental work bearing on this question was very limited. In my laboratory experiments have been performed to ascertain whether three strains of yeast were capable of growing continuously in the absence of inositol, and whether they were able to synthesize the substance during growth. That yeasts grown in the absence of inositol might be practically free from this substance was considered as a possibility. The results showed that the yeasts tested can grow from a minute seeding without inositol and all evidence pointed to the presence of substantially a normal amount of inositol in the final yeast crops. It appears, therefore, that inositol performs some important function in yeast physiology and that when yeasts grow in its absence they do so by virtue of their ability to synthesize it from other materials in the culture medium. Janssens (1935), however, found inositol to be indispensable for the growth of Wildiers's yeast, and in a recent study (R.J.W., Eakin *et al.* 1940) it appears that "old process" yeast may require inositol for continued reproduction.

The general biological significance of inositol is obscure, though its widespread occurrence in tissues (both free and in the form of phytic acid) is well recognized. It is said to disappear in ripening seeds and to appear when sprouting occurs. In animal tissues, its presence and distribution has never been interpreted successfully.

One of the difficulties which has beset the study of the physiological function of inositol is the lack of a satisfactory method for its determination. Needham (1923) found the colour reactions of no value for quantitative study and determined inositol by isolating it in semi-pure condition and running a micro-combustion on the product. Young (1934*a*, *b*) also carried through a time-consuming isolation procedure and titrated the final material with potassium iodomercurate solution. This was applied by him to a few animal tissues, but not to plant materials. In my laboratory it proved not to be applicable to yeast, since other polyalcohols seemed to be present and increase the value obtained.

Yeast growth will doubtless ultimately serve as a tool whereby inositol can be determined biologically and probably with reasonably high accuracy; also in micro- and "submicro"-amounts. Eastcott (1928) carried out a few experiments along this line and Kögl & van Hasselt (1936*a*) determined inositol in yeast concentrates using the same principle. However, neither of these workers present evidence or claims to a method which can be used in testing various tissues. Stout (1938), working in my laboratory, found it difficult to prepare a basal medium which was improved only by the addition of inositol. She did, however, succeed in developing a method of some value, and applied it to various tissues. As more yeast nutrilites are recognized and become available it becomes increasingly feasible to determine inositol by its effect on yeast growth. Extracts containing inositol will owe their effects to it alone when the basal medium contains all the other requisites for rapid growth.

(2) THIAMIN

This vitamin was first isolated in minute amounts by Jansen & Donath in 1926. Years later it was obtained in amounts sufficient for extended chemical study (R. R. Williams *et al.* 1934). This resulted in the determination of its structure and synthesis. The whole subject of vitamin B_1 has recently been reviewed (R. R. Williams & Spies, 1938) and need not be treated fully here, except briefly as it is related to yeast growth.

The early suggestions regarding the effectiveness of the antineuritic vitamin in yeast growth stimulation have been mentioned. Since "old process" yeast used in my first investigation (R.J.W. 1919) is now known to be highly dependent upon an outside source of thiamin under the conditions employed, it is certain that the response obtained at that time was actually due in part to thiamin, and that when thiamin was removed by fuller's earth adsorption or phosphotungstic acid precipitation the extract lost most of its effectiveness for this reason.

The first conclusive evidence that the antineuritic vitamin is effective in yeast growth stimulation was presented by R.J.W. & Roehm (1930), who tested the crystalline vitamin as prepared by Jansen & Donath and found it effective in concentrations as low as 0·00001 mg. per ml. of culture medium, when supplemented by a residue from rice bran which was unadsorbed by fuller's earth. The yeasts used were no. 578 of the American Type Culture Collection and "old process" yeast. The experiments have been repeated in various ways in my laboratories many times both with the natural and the synthetic vitamin.

Several other yeasts are relatively little affected by thiamin under the usual growth conditions (R.J.W. & Saunders, 1934; R.J.W., Eakin, *et al.* 1940). Closely related, however, is the observation of Schultz *et al.* (1937*a*) that yeast fermentation (even in the absence of growth) is greatly enhanced by the presence of thiamin. This test with modifications has been developed as a quantitative method for determining minute amounts of thiamin (Schultz *et al.* 1937*b*).

Peskett (1927*b*) showed that the antineuritic vitamin may be synthesized by yeast. The work of Pavcek *et al.* (1937) demonstrated that certain yeasts are able to synthesize thiamin during growth, but that the thiamin content of yeast grown on a "synthetic" medium was low. Schultz *et al.* (1938) showed that certain yeasts which respond to thiamin are completely activated by the thiazole and pyrimidine cleavage products of the vitamin. When yeast is grown on a medium well supplied with thiamin or the appropriate thiazole and pyrimidine intermediates, the thiamin content of the yeast crop is greatly increased.

In a recent study involving growth over long periods from small seedings R.J.W., Eakin *et al.* (1940) have shown that even "old process" yeast, which under certain growth conditions is highly responsive to thiamin, can grow continuously in its absence and must therefore be capable of synthesizing it from simple constituents of the medium. Many other yeasts are evidently able to carry on this synthetic process more rapidly.

The extreme importance of thiamin as a constituent of tissues in general and its effectiveness on numerous types of organisms need not be stressed here.

(3) BIOTIN

This substance was discovered and isolated in the form of the methyl ester (which is biologically active) in the laboratories of Kögl (1935). Its empirical formula is stated to be $C_{11}H_{18}O_3N_2S$ (Kögl, 1937). In free condition it is amphoteric with predominant acid properties.

The outstanding facts regarding it appear to be the extraordinarily small amounts present in the richest available sources, the extremely small doses which suffice for yeast growth stimulation and the fact that in connexion with its discovery relatively high seedings of yeast were used in contrast to most "bios" investigations in which the seedings of yeast have been small.

In the fractionation procedure which has been reported, Kögl & Tönnis (1936) started with 250 kg. of Chinese egg yolk containing 2×10^9 "S.E." units and carried out an extensive series of procedures involving removal of extraneous material with acetone, alcohol, lead acetate, mercuric chloride, brom-picrolonic acid and adsorption (repeated) of the active substance on charcoal, its precipitation (repeated) with phosphotungstic acid, its esterification, high vacuum distillation and crystallization. The final yield after sixteen steps was 1·4% of that originally present, which is a remarkably good recovery. The total amount of crystalline material, however, was only 1·1 mg., which showed that the original batch of egg yolk contained only 80 mg. of biotin. According to biotin determinations by a method recently devised by Snell *et al.* (1940*a*), liver (autolysed) is a richer source than egg yolk (though not a practical one because of its admixture with much other water soluble material) and a water extract of manure was even richer than liver autolysate. According to these studies the latter extracted material calculated on a dry-weight basis contained 16 μg. of biotin per g. The biotin content of this relatively very rich source is less than the thiamin content of rice polish. Bakers' yeast contains only about 0·12 μg. of biotin per g., whereas it may contain 20 μg. or more of thiamin per g.

Biotin, in keeping with its low concentration in tissues, possesses extraordinary activity in minute doses. Thus, according to the results of Kögl, one "S.E." unit, i.e. the amount necessary to add to a 2 ml. culture to cause a 100 % increase in the yeast crop (Rasse M., 5 hr.) is 0·00004 μg. This is 2 parts per 10^{11} parts of medium. Kögl states that the substance is detectable even in a concentration equivalent to 1 part per 4 × 10^{11} parts of medium. In the recent test of Snell *et al.* (1940*a*) involving another yeast the effects of small amounts of biotin are perhaps even more striking since four-fifths of the lowest concentration above caused a galvanometer deflexion (measuring turbidity due to yeast) of 22 while the blank showed a deflexion of 16 divisions.

In the test of Kögl the yeast seeding is 0·1 mg. per ml., the growth takes place for 5 hr., and the multiplication of the yeast under testing conditions is not more than about onefold. Under these conditions the most important limiting factor for growth (of the strain used) is biotin. The various other substances which under appropriate conditions may affect yeast growth are sufficiently well supplied in original seed yeast and a deficiency of these does not become apparent during the propagation of one new generation. It is in general dangerous to draw inferences from results obtained on one strain of yeast and apply them to a different strain. In this case, however, it appears that in the test of Snell *et al.* (1940*a*) the nutrilites other than biotin which are necessary for extended (rapid) multiplication are supplied in the basal medium and for this reason the biotin becomes a limiting factor. Particularly interesting is the demonstration cited, showing that in the absence of vitamin B_6 biotin is almost without effect (under the conditions used), but that the addition of a minute amount of B_6 causes the biotin effect to become at once apparent and striking.

In the studies of R.J.W., Eakin *et al.* (1940) it was shown that the three diverse strains of yeast tested (all of which are or have been grown commercially for baking purposes) are able to grow from a small seeding continuously in the absence of biotin, even though they respond to it strikingly under appropriate conditions. Yeasts grown in the absence of biotin contain the substance and it is evident that synthesis takes place during growth. The stimulative effect of biotin is due, not to the fact that an exogenous source is required, but that its rate of synthesis may limit growth.

Two findings regarding biotin were reported by Snell *et al.* (1940*a*): first, that it is destroyed by nitrous acid as rapidly as are α-amino acids under similar conditions and more rapidly than β- or γ-amino acids; second, that whereas an extract of fresh liver contained comparatively little biotin, autolysis frees the substance so that autolysed liver is a rich source. Evidently biotin exists in liver tissue in a combined insoluble form.

The distribution of biotin in animal tissues has been studied by Kögl & van Hasselt (1936*b*). They found most of the tissues from dog, cow, and steer calf to be relatively uniform in content, averaging about 0·007 μg. per g. of fresh tissue. The intestinal contents of the one cow examined were found to be very rich, containing up to 0·05 μg. per g. This finding is in accord with the high biotin content of the extract of cow manure referred to above. The fresh livers of the animals yielded about the average amount, namely 0·007 μg. per g. According to the results of Snell *et al.* (1940*a*), autolysis increases the extractable biotin from liver more than 20 times. The tissues of a leghorn hen were on the average about three times as rich as those of the mammals. The kidney was high in content, about 0·1 μg. per g., as were liver and egg follicle (18 g.) 0·07 μg. per g.

Plant tissues (Kögl & Haagen-Smit, 1936) on an air dry basis yield by simple extraction from about 0·02 μg. per g. with asparagus and corn up to about 0·2 μg. in the case of parsley and garden cress.

Biotin is very stable compared with the other known yeast nutrilites inasmuch as it will withstand refluxing or even autoclaving in an acid or alkaline condition. As indicated, the methyl ester of biotin is practically equivalent in its effect on yeast to the free acid.

Biotin is not only effective as a nutrilite for yeast but also for various fungi and bacteria (Kögl & Fries, 1937; Kögl & van Wagtendonk, 1938; Robbins & Schmidt, 1939*a*), and is the only nutrilite requirement of certain butyl alcohol producing anaerobes (*Clostridia*) (Snell & R.J.W., 1939). It is found to be identical with the "coenzyme R" of Allison & Hoover (1934) (Nilsson *et al.* 1939; West & Wilson, 1939). It is also effective in the stimulation of growth of green plants (Kögl & Haagen-Smit, 1936).

Up to the date of writing this review, biotin has not been found to be significant for animal nutrition, though evidence very recently presented (György *et al.* 1940) shows that it may be identical with "vitamin H", the factor which cures dermatitis associated with egg-white injury. The fact that it is widespread in animal tissues, rather abundant in milk, and is found in a "bound" condition in liver, all point to the probability that it is significant in animal physiology. Its presence in relatively high concentrations in intestinal contents and manure suggests that it may here be predominantly a product of bacterial action, and animals may not ordinarily become deficient with respect to it, because of its formation in the intestine.

(4) PANTOTHENIC ACID

The first intimations as to the existence of this substance are found in the publications of R.J.W. & Bradway (1931) and R.J.W. & Truesdail (1931), in which was set down the opinion that the nutrilite for "Gebrüder Mayer" yeast, under the test conditions used, was a single substance. This was followed by a preliminary announcement (R.J.W. *et al.* 1932) of the evidence that the nutrilite for this yeast is of universal biological importance, and in 1933 (R.J.W. *et al.*) an extended discussion of its properties and the evidence for its existence.

The demonstration of the acid properties of this nutrilite came about through the introduction of a new tool, namely, fractional electrical transport in a high potential field without the use of membranes. This was first used by R.J.W. & Truesdail (1931), R.J.W. *et al.* (1933), R.J.W. & Moser (1934), and was further refined and described by Williams (1935). It was found that in order to cause the migration and separation of relatively weak acids and bases of low molecular weight, high potentials were necessary and that membranes had to be avoided because of electro-osmose. The essential of this method has been fruitfully developed by Tiselius (1937) in connexion with the study of the electrical migration of proteins.

It was found (R.J.W. *et al.* 1933) that when extracts from widely different biological sources representing Chordata, Arthropoda, Echinodermata, Mollusca, Annelida, Platyhelminthes, Myxomycetes, Bacteria, Fungi, Algae and Spermatophytes, were tested and subjected to exhaustive electrolysis in an eight-cell apparatus, the nutrilite effective for this yeast was always present and migrated like an acid of about the same strength. The nutrilite from various sources had diffusion constants about the same (indicating a molecular weight of about 200) and experiments involving hydrogenation, oxidation, treatment with nitrous acid, tests of heat stability in acid, neutral and basic media, esterification experiments and solubility tests indicated that the same acid (probably with more than one hydroxyl group) was present in the diverse tissues. Later experiments showed that it appeared to be universally distributed in animal tissues (Rohrmann *et al.* 1934). It was designated "pantothenic acid", the name being derived from the Greek meaning *from everywhere.*

Subsequently, a more exhaustive study of the electrolytic behaviour of pantothenic acid in comparison with acids of known strength (R.J.W. & Moser, 1934), led to the conclusion that pantothenic acid has an ionization constant approximately that of gallic acid ($3{\cdot}9 \times 10^{-5}$) though slightly lower. From this it was deduced that it could not be an α-hydroxy acid since all α-hydroxy acids have constants 10^{-4} or higher. At this time it was thought that pantothenic acid was free from nitrogen and without amphoteric properties. Later (R.J.W. *et al.* 1939) it was reported to possess very feeble basic properties, because in refined experiments the acid migrated somewhat away from the anode cell. The physiological effect of the substance disappears when the mixture containing it is subjected to esterification conditions, but reappears on mild acid or basic hydrolysis. The ester of pantothenic acid formed as above migrated under high potential like a very weak base, indicating the presence of nitrogen.

An outstanding point of interest in the pantothenic acid investigation is the fact that so much regarding its structure was determined before it was obtained in pure condition. In fact, the complete structure was known and the physiologically active substance was synthesized before pantothenic acid or its salts or other derivatives (excluding cleavage products) were obtained in condition such as to yield the correct analyses. The most potent material obtained previous to synthesis was the calcium salt preparation described by R.J.W. *et al.* (1939). This (on the basis of the β-alanine yields, Weinstock *et al.* 1939) was adjudged to be not more than 90% pure. Its impurity was later further shown by the fact that whereas its analysis led to the molecular formula (for the calcium salt) of $(C_8H_{14}O_5N)_2Ca$, the correct formula is $(C_9H_{16}O_5N)_2Ca$ (R.J.W. & Major, 1940).

The discovery of the presence of β-alanine as a cleavage product of pantothenic acid was preceded by the finding that β-alanine itself is effective as a yeast nutrilite in very minute doses (R.J.W. & Rohrmann, 1936). Its effectiveness is characterized by being small in a medium containing a relatively high concentration of asparagin or aspartic acid, but relatively large when these substances are left out of the medium or their concentration diminished. It also shows the very interesting behaviour of being slightly but definitely inhibitive of yeast growth in doses much lower than can be detected by its stimulative effect. The physiologically active substance formed by cleavage of pantothenic acid (in various stages of concentration) was first identified by the above physiological effects, and was later proved to be present by other means and finally isolated as the β-naphthalene sulphonyl derivative (Weinstock *et al.* 1939).

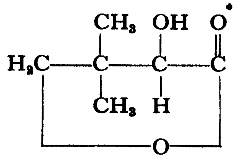
The finding that β-alanine was effective for yeast growth stimulation was not entirely accidental. We first became interested in this substance because of the peculiar efficacy of aspartic acid (or asparagin) as a yeast nutrient (R.J.W. & Rohrmann, 1936). These findings have never been reported in detail, but we were led to regard aspartic acid as peculiarly effective, compared with other amino acids, for all of the yeasts tested. Recognizing that aspartic acid was unique among the common amino acids in that it is a β-amino acid (as well as an α-amino acid), we thought it desirable to test the effects of decarboxylated aspartic acid, i.e. β-alanine, and it was synthesized in our laboratory especially for this purpose. In our first tests the dosages of β-alanine used were too high and its extraordinary properties escaped our notice temporarily. Later, when minute doses were used, its striking stimulative effect was observed.

β-Alanine is doubtless present in small amounts in meat extracts, etc., since it may arise by the hydrolysis of carnosine and anserine (or in traces from pantothenic acid). The first two unsplit peptides are, however, ineffective for yeast growth stimulation and we have no experimental evidence to indicate that β-alanine has been a significant constituent of yeast culture media. Its importance in this connexion probably lies primarily in the fact that it may, in artificial media, be a precursor of pantothenic acid.

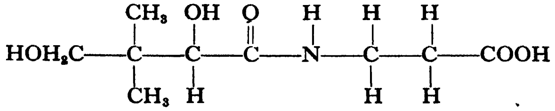
The study in my laboratory, based entirely on partially purified material and to a considerable extent on material which was highly impure, led to the proposed formula $C_8H_{15}O_5N$ for the free pantothenic acid and indicated that it was a peptide-like combination between a saturated dihydroxy acid and β-alanine (Weinstock *et al.* 1939). It was also found (Mitchell *et al.* 1940*b*) that the non-β-alanine portion of the molecule lactonized after cleavage, and that in this portion of the molecule a hydroxyl group was in the α-position. Since the lactone formed readily, the γ-position for the second hydroxyl group suggested itself. This probability was strengthened by the fact that the physiological activity of pantothenic acid was readily destroyed by acidic acetone, acetaldehyde or benzaldehyde, which in this case could take place through the formation of an acetal with a six-membered ring, and would require the formation of a seven-membered ring in case the second hydroxyl were in the δ-position. Oxidation studies showed that no two hydroxyl groups could be in adjacent positions and the absence of a β-hydroxyl group was demonstrated.

It was also shown that the lactone formed by cleavage of pantothenic acid could be condensed with a β-alanine ester to bring about a partial synthesis of pantothenic acid (R.J.W. 1938) and that three synthetic α-hydroxy-γ-lactones derived from valeric acids yielded products with some slight physiological activity (Mitchell *et al.* 1940*b*).

At this time I accepted a new post and moved my laboratory, and a co-operative arrangement was made with the Merck Research Laboratories to continue the study. In these laboratories large amounts of pantothenic acid concentrates were prepared, from which the lactone was obtained in pure form, and its structure determined by degradation and synthesis to be (I).



(I)



$$\mathrm{HOH_2C{-}\overset{CH_3}{\underset{CH_3}{C}}{-}\overset{OH}{\underset{H}{C}}{-}\overset{O}{\overset{\|}{C}}{-}\overset{H}{N}{-}\overset{H}{\underset{H}{C}}{-}\overset{H}{\underset{H}{C}}{-}COOH}$$

(II)

This was condensed with β-alanine to yield the physiologically active pantothenic acid (II) (R.J.W. and Major, 1940; Stiller *et al.* 1940*a*). This was obtained in (+) and (−) forms and the (−) form found to be physiologically inactive (Stiller *et al.* 1940*b*). Subsequently Mitchell *et al.* (1940 *a*) reported the physiological activity of "hydroxy pantothenic acid", and R.J.W., Mitchell *et al.* (1940) described a simple method for condensing the two parts of the molecule.

In the meantime Jukes (1939*a*) had obtained samples of pantothenic acid from my laboratory and showed it to be identical with the "chick anti-dermatitis factor". Woolley *et al.* (1939*a*, *b*) gave evidence pointing in the same direction and reported a partial synthesis similar to that referred to above, though not making use of the lactone character of the non-β-alanine portion of the molecule.

The isolation of pantothenic acid from natural sources offers unusual difficulties. (1) It is predominantly an acid rather than a basic substance and hence the techniques and precipitants extensively useful with nitrogenous bases cannot be applied except indirectly. (2) The richest convenient source (liver) contains approximately 40 parts per million, and hence 250 kg. of liver, after an extended process, yielded only about 3 g. of crude (approximately 40%) material from which the remaining impurities were removed with great difficulty. (3) The substance is highly hydrophilic and we have yet to find any salt or simple derivative of it which is not highly soluble in water. (4) Due, presumably, to the presence of several types of functional groups, even after purification the substance has failed to crystallize. In this respect it appears to behave somewhat like β-hydroxy-glutamic acid, which, according to Dakin (1918), makes up 10% of casein, yet is not available on the market at any price. (5) Pantothenic acid is itself unstable and can be handled safely only as a neutral salt. It is readily broken down by acids or bases and is particularly susceptible to destruction by acidic alcohol. (6) Unlike riboflavin and ascorbic acid, pantothenic acid could be tested for by biological tests only, since it has no colour, reducing properties or other known outstanding property which can be used as a guide during its isolation. (7) Unlike the amino acids and biotin, pantothenic acid has not been purified successfully by distillation of its ester.

Its concentration to a point where it was about 90% pure was accomplished starting with autolysed liver. Fuller's earth adsorption was used to remove bases, after which the active principle was adsorbed on Norite and eluted with ammonia solution. It was then converted to its brucine salt and subjected to an elaborate fractionation involving a distribution of the salts between water and chloroform. The material was subsequently converted to the calcium salts and further fractionated using mercuric chloride (to remove impurities) and various solvents—ethanol, isopropyl ether, acetone and pyridine. As the purification proceeded it became increasingly difficult to make progress and at no time was a crystalline salt or other derivative obtained.

While the "Gebrüder Mayer" strain of yeast was used as the test organism in a majority of the earlier studies on pantothenic acid, it was found to be an important nutrilite for all of the yeasts tested (R.J.W. & Saunders, 1934; R.J.W., Eakin *et al.* 1940). "Gebrüder Mayer" yeast appears not to be able to synthesize this nutrilite and for other yeasts its rate of synthesis may be a limiting factor for growth, especially when extensive multiplication is involved. In the short-period tests of Kögl, in which only one new generation of yeast is propagated, pantothenic acid does not appear to be a limiting factor for the growth of any yeast which we have tested.

Certain moulds and fungi synthesize pantothenic acid readily during growth (R.J.W. *et al.* 1933) and for some it serves as nutrilite without being an indispensable constituent (Mosher *et al.* 1936).

All lactic acid producing bacteria tested, and some propionic acid bacteria were shown to require an acidic substance later identified as pantothenic acid. This discovery of Snell *et al.* (1938, 1939) was the basis for an extended study which resulted in the concentration of pantothenic acid simultaneously with the work carried on in my laboratory (Snell *et al.* 1937, 1939). Certain lactic acid producing bacteria are responsive to pantothenic acid in extremely small doses. In one experiment (Sue Stanbery in my laboratory) 0·00004 μg. of pantothenic acid per ml. caused a fourfold increase of bacterial cells (*Streptococcus lactis*). This concentration is about the same as the least concentration of biotin which is effective for yeast. Various strains of diphtheria bacilli (Mueller & Klotz, 1938), hemolytic streptococci (Subbarow & Rane, 1939; McIlwain, 1939; Woolley & Hutchings, 1939; Krauskopf *et al.* 1939), and *Proteus* (Pelczar & Porter, 1940) also require pantothenic acid for growth. In the case of the lactic acid bacteria β-alanine cannot serve as a nutrilite. For certain diphtheria organisms, however, β-alanine can take the place of pantothenic acid and presumably is useful as a precursor of it (Mueller & Klotz, 1938; Evans *et al.* 1939*a*, *b*). Woolley (1939) has found one strain of a hemolytic streptococcus which responds to the non-nitrogenous fragment of pantothenic acid.

Certain protozoa were found to be stimulated by the presence of pantothenic acid (Elliot, 1935). These experiments were conducted before pantothenic acid in highly concentrated form became available and the subject should be reinvestigated using pure pantothenic acid and other nutrilites as well.

Green plants (liverworts, alfalfa) are stimulated by pantothenic acid (R.J.W. & Rohrmann, 1935; McBurney *et al.* 1935) which appears to be mobilized, for example, during the sprouting of a potato. The stimulating effect of pantothenic acid on the respiration of plant tissues is readily demonstrated (Pratt & R.J.W., 1939). As young alfalfa seedlings become green and grow, their pantothenic acid content increases markedly, showing that this substance is synthesized by green plants.

The requirement of chicks for pantothenic acid has been mentioned above. They are readily depleted on a diet which has been subjected to a dry heat treatment, and highly concentrated pantothenic acid when added to such a diet induces approximately normal growth (Jukes, 1939*a*). The pantothenic acid requirement of young growing chicks is placed by Jukes (1939*b*) at about 1·4 mg. per 100 g. of diet. The tissues of chicks on such a deficient diet contain from 30 to 60 % the normal amount when calculated on a weight basis (Snell *et al.* 1940*b*).

Phillips & Engel (1939) have reported that pantothenic acid is necessary for the maintenance of the normal intact structures of the spinal cord in the chick, and that thiamin, nicotin-amide and riboflavin were not effective in preventing the spinal cord pathology. Bauernfeind & Norris (1939) kept laying hens on a heated diet practically free from pantothenic acid for 28 weeks without any macroscopic evidence of dermatosis and without any effect on egg production or mortality. However, the hatchability of the eggs dropped from over 60 % to less than 3 % and the chicks hatched from the deficient eggs showed very early symptoms of dermatitis. It thus appears probable that an adult hen retains to a marked degree the pantothenic acid present in her own tissues. Preliminary unpublished experiments in my laboratory (C. E. Meyer) indicate that rat tissues likewise do not readily become depleted.

That pantothenic acid is required for the nutrition of rats is indicated by experiments in various laboratories (Subbarow & Hitchings, 1939; Oleson *et al.* 1939; György *et al.* 1939; R. R. Williams, 1939; Black *et al.* 1940). Elvehjem and co-workers have obtained indications that it is required by dogs (McKibbin *et al.* 1939). It seems altogether probable that it is a nutritional requirement of all animals. Bacterial production in the gastro-intestinal tract is a factor in some cases (McElroy & Goss, 1939).

There are three general methods for assaying tissues or extracts for pantothenic acid. They involve the growth response of chicks (Jukes, 1939*b*), lactic acid bacteria and yeast, respectively (Snell *et al.* 1940*a*; Pennington *et al.* 1940). The first, while cumbersome and expensive, should be of value in connexion with tissues in which pantothenic acid may be in a combined non-extractable form. The second and third are available for use with extracts.

In the liver, and to an unknown extent in other tissues, pantothenic acid is definitely in combined form. An extract of fresh liver yields only a small fraction of the amount which is obtained after autolysis. This fact is doubtless related to the observation above, that animal tissues do not rapidly lose the pantothenic acid which they contain when the animal is on a pantothenic acid-free diet.

The use of the yeast test method and a test involving the use of *Lactobacillus casei* ε have been investigated and compared (Snell *et al.* 1940*a*; Pennington *et al.*

1940), and the latter has been found preferable partly because the yeast is more susceptible to inhibiting substances which are likely to be in extracts. At high dosages yeast also responds to β-alanine and other nutrilites.

(5) VITAMIN B_6 (PYRIDOXIN)

This vitamin was first clearly recognized as a substance preventing dermatitis in rats by P. György in 1935. It was isolated in crystalline form by Keresztesy & Stevens (1938) and Lepkovsky (1938), and was synthesized by Harris & Folkers (1939). Its structure is that of 1-methyl-2-hydroxy-3, 4-dihydroxymethyl pyridine.

It was reported simultaneously by Schultz *et al.* (1939) and Eakin & Williams (1939*a*) to be stimulatory for yeast growth. The basal media used in the two sets of tests were different and the crystalline vitamin had biological activity in doses down to 0·0001 μg. per c.c. In order for the B_6 response to be obtained, the medium must contain other nutrilites, especially pantothenic acid (or β-alanine) and biotin. In the discussion of biotin we have already alluded to the fact that vitamin B_6 has a profound effect on the response of at least one yeast to biotin.

Vitamin B_6 seems to be either dispensable so far as these yeasts are concerned, or more likely, can be synthesized by the yeasts during growth. In long-period tests no substantial difference in crop was observed whether or not vitamin B_6 was supplied in the medium. It has been found to be a "growth substance" for excised tomato roots (Robbins and Schmidt, 1939*b*).

(6) MISCELLANEOUS COMPOUNDS

Space will not be taken here to discuss the rather numerous compounds which have been observed to have appreciable stimulating effects upon yeast growth. Most of these substances are effective when used in concentrations greater than those required for the nutrilites discussed above, and with possibly few exceptions the maximum effects observed have not been large as compared with those described above.

The substances may be classified into two groups: first, those which contribute building stones for known chemical constituents of yeast, and second, those which for other and unknown reasons have a stimulatory effect.

Among those which probably contribute to known yeast constituents are: aspartic acid, leucine and other amino acids (Nielsen & Hartelius, 1937) which may be useful for protein synthesis; uracil (Snell, 1940), which presumably enters into the synthesis of nucleic acid; ethanolamine (R.J.W. 1939*b*), which is present in cephalins; nicotinamide and nicotinic acid (Rohrmann, 1936), and riboflavin (Mitchell, 1939), which are coenzyme constituents.

To these might be added various synthetic pyridine acids which like nicotinic acid and nicotinamide were found in my laboratory (Rohrmann, 1936) to be slightly active.

Among the other compounds which have slight effects for unknown reasons are indole butyric acid (Rohrmann, 1936), carotene (Pratt, 1938), choline (Mitchell, 1940), acetyl choline (Mitchell, 1940), colchicine (Richards, 1938), deuterium oxide

(Richards, 1934), dibenzanthracene derivatives (Cook *et al.* 1938), nitrophenols (Field *et al.* 1934), and follicular hormone (Lund, 1936). The above list is not exhaustive.

(7) UNIDENTIFIED SUBSTANCES

A recent study by R.J.W., Eakin *et al.* (1940) has shown that for three strains of yeast a mixture of the five principal nutrilites mentioned above will not induce growth at a rate comparable with that obtained when a liver extract is added to the medium. This was interpreted to mean that in liver extract there are unidentified substances which, in addition to those known, are effective in promoting the more rapid growth of yeast.

It seems premature at this time to discuss the various substances and their action. Most of the more or less hypothetical substances are poorly characterized, have not been obtained in concentrated form, and their existence has not been confirmed in more than one laboratory. Included among such substances are the "biotic acid" of Eakin & R.J.W. (1939*b*), "biosterol" of Devloo (1938), Bios IIA (pantothenic acid?), Bios IIB (biotin?) and Bios V (thiamin?) of the Lash Miller Laboratories, the Bios III mentioned by Kögl, and the fermentation and growth-stimulating substance isolated by Yamafuji & Ohtsu (1939). When these substances have been purified and studied adequately, preferably in more than one laboratory, it will be possible to evaluate them and their growth-stimulating effects.

This brief discussion of unidentified factors should include a mention of some of those which have been found to affect the enzymes and enzyme systems of yeast, for example, the "Z" factors of von Euler, the factor studied by E. W. Miller (1921) which stimulates invertase activity and the interesting substances which stimulate the fermentation of maltose (Blish & Sandstedt, 1937). It is quite possible that a considerable overlapping between these enzymatic factors and the nutrilites will eventually be discovered.

III. GENERAL BIOLOGICAL SIGNIFICANCE

A full discussion of the bearing of the nutrilites on the problems of yeast physiology or of general physiology is beyond the scope of this review. Physiologically active principles must first be identified before they can be studied effectively and some time must elapse before the significance of some of the recently discovered nutrilites for yeast can be understood.

(1) INTERRELATIONS

The only study that has been made of the interrelations of the five nutrilites discussed above is that of R.J.W., Eakin *et al.* (1940). The growth curves of three distinct strains of *Saccharomyces cerevisiae* (all of which have been propagated commercially for baking purposes) in media containing various of the nutrilites are presented in Figs. 1, 2 and 3. The concentrations of the different nutrilites, when present, were the same in all cases, and constituted what seemed from preliminary experiments to be favourable concentrations.

Three phenomena of particular interest become apparent as a result of such studies. The first is the *supplementary* effect of one nutrilite for another. This has often been observed and is in line with the general conception of limiting factors. Each nutrilite under the proper conditions may be a limiting factor for growth.

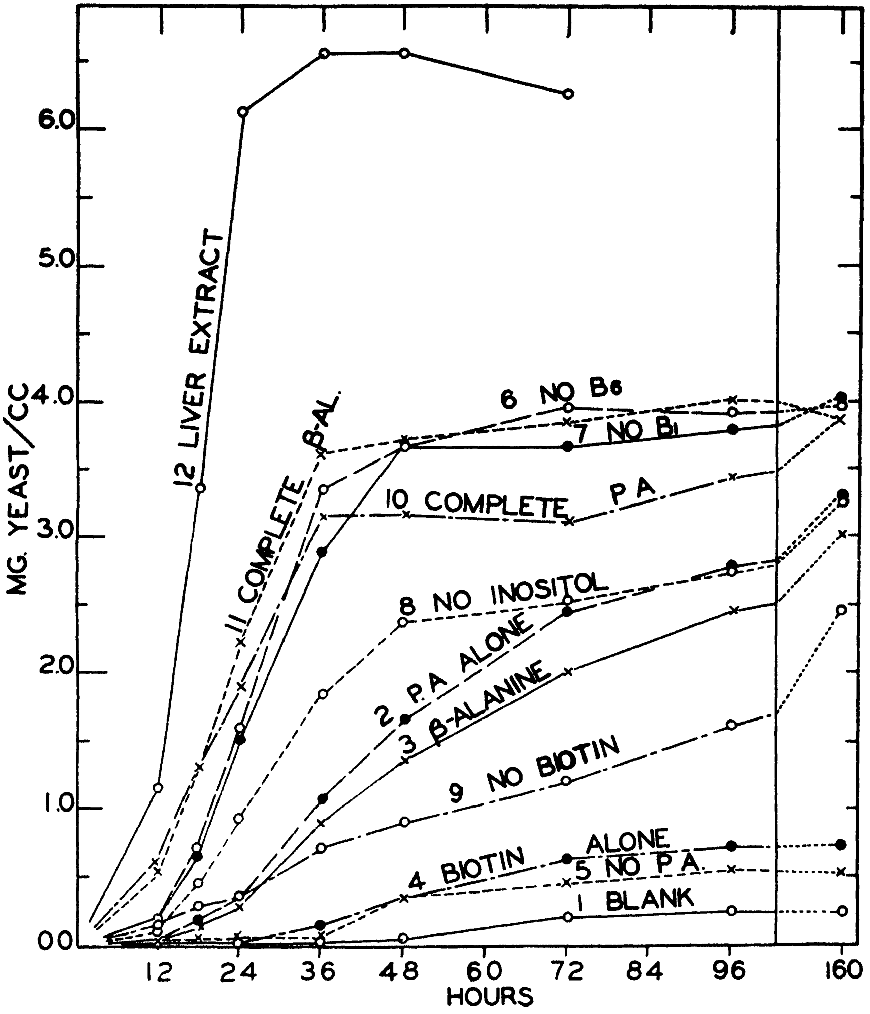


Fig. 1. Growth curves of "Gebruder Mayer" yeast.
(In Figs. 1, 2 and 3 "No biotin" etc. refer to media in which the nutrilite indicated is the only one of the five lacking.)

The *replaceability* of one nutrilite for another is contrary to what might be expected, yet it becomes apparent from Fig. 3 that when either pantothenic acid (or β-alanine) alone or biotin alone is introduced into the medium, the growth of this yeast is materially greater than in the blank. It should be added that neither of these nutrilites is strictly indispensable for the growth of the yeast (Fleischmann baker's), and that the effects are qualitatively different. Pantothenic acid shows its effect very early, but growth continues slowly thereafter. The effect of biotin alone first appears after 36 hr., but growth is rapid thereafter. This can be interpreted to mean that in the presence of biotin this yeast eventually develops the ability to synthesize pantothenic acid relatively rapidly, but that the reverse does not hold. A somewhat similar replaceability of biotin and pantothenic acid is also noted in the case of the "old process" yeast (Fig. 2). Biotin alone has no effect until the end of 72 hr., when continued rapid growth begins. Pantothenic acid shows its effect much earlier and in this case proceeds to a higher level. This yeast evidently requires a considerable period before it synthesizes thiamin rapidly enough for appreciable growth.

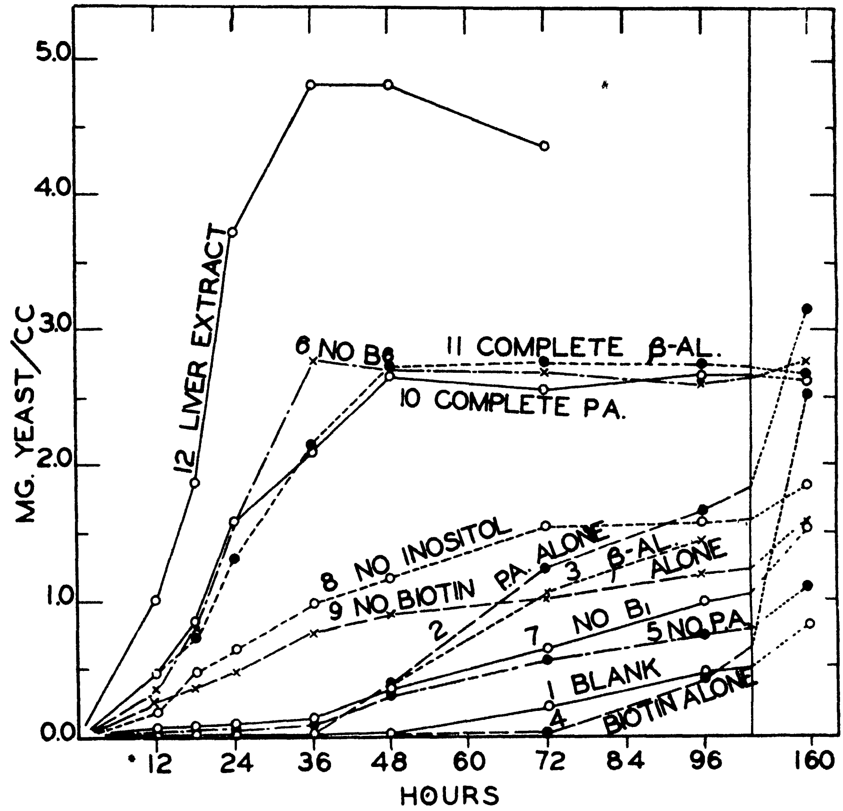


Fig. 2. Growth curves of "old process" yeast.

In Fig. 1 it appears that pantothenic acid is indispensable for the continued growth of "Gebrüder Mayer" yeast beyond about 72 hr., and that biotin cannot take its place.

Replaceability of one nutrilite for another was previously observed in my laboratory in the case of a pathogenic mould *Trichophyton interdigitale*. In this case no one of the nutrilites may have been absolutely indispensable (Mosher *et al.* 1936).

Another phenomenon which has been observed repeatedly in my laboratory is that of a *balance* or even an antagonism between various nutrilites. For example, when a minute dose of vitamin B_6 was introduced into a medium containing no biotin, growth was suppressed. When added to a similar medium containing biotin, vitamin B_6 caused a large increase in growth. Thiamin and pantothenic acid enter into the complex balance also. When thiamin and vitamin B_6 are absent from the medium and pantothenic acid is present in excess, biotin produces a good response. When thiamin is present, vitamin B_6 absent and pantothenic acid is in *low* concentration, biotin is relatively ineffective. When thiamin is present and pantothenic

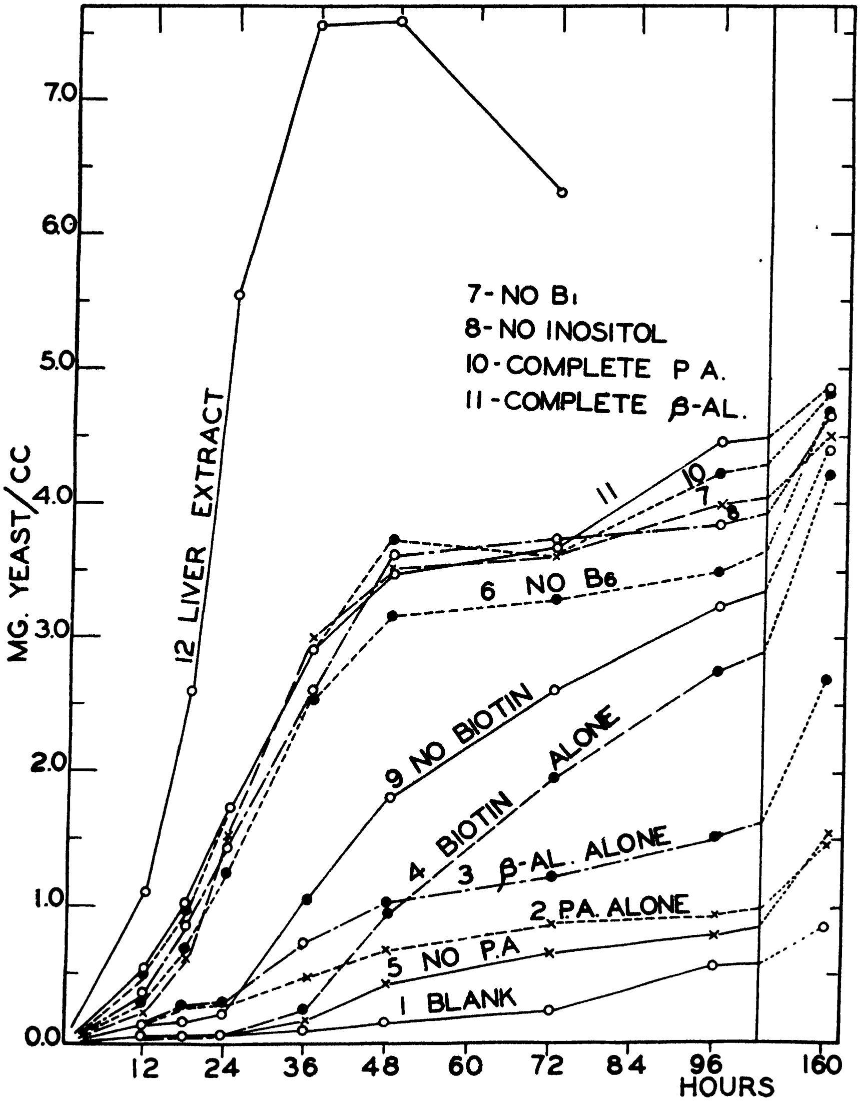


Fig. 3. Growth curves of Fleischmann baker's yeast.

acid (or β-alanine) is abundant, vitamin B_6 is necessary for the biotin effect. These statements apply to tests involving low seeding and 16-hr. growth periods of Fleischmann's baker's yeast. Many other complicated relations may be observed in the study of growth curves similar to those shown in Figs. 1–3.

(2) RELATION TO VITAMINS

It is apparent that there is a close connexion between the yeast nutrilites and the vitamins which play such important roles in animal nutrition. Thiamin, pantothenic acid and vitamin B_6 are both yeast nutrilites and vitamins. Biotin may be a vitamin. It is possible that inositol may be found to be a factor in animal nutrition, though this seems unlikely. Other yeast nutrilites which are as yet undiscovered or unidentified may be identical with undiscovered components of the "vitamin B complex".

The close relationship between yeast nutrilites and vitamins suggests the likelihood of complex balance relationships among the vitamins in animal nutrition. There are indeed abundant suggestions of such balances in the study of vitamins themselves, but it may well be that such balances will be profitably studied first in connexion with yeasts and micro-organisms. The study of yeast nutrilites also suggests the possibility that "vitamins" exist which are helpful to, but not indispensable for, animal growth. Rose (1938) has observed that this is characteristic of the amino acid arginine, but no clear-cut case appears to have been observed among the vitamins. The replaceability of one vitamin for another, if such exists, will be limited to those vitamins, if any, which are dispensable.

(3) PROBABLE FUNCTIONING IN ENZYME SYSTEMS

The most plausible hypothesis regarding the function of the nutrilites which yeasts require for rapid growth is that they enter into enzyme systems. Thiamin pyrophosphate is known to be an important coenzyme (cocarboxylase), so that the case of thiamin rests on a firm foundation. Pantothenic acid is probably of the nature of a coenzyme since it affects fermentation by non-living as well as living systems (Pratt & R.J.W., 1939). Biotin is effective in such very high dilution that a catalytic function seems inescapable. Inositol remains an enigma and vitamin B_6 has not yet been studied fully in this connexion.

If the hypothesis of the coenzymic function of the nutrilites is adopted, the replaceability of one nutrilite for another, in long growth tests, finds an explanation in the possibility that more than one enzyme system (or set of systems) may be available for the production of energy, and that other systems may be built up more readily if the necessary building stone for one system is furnished.

(4) RELATIONSHIP TO NUTRIENTS

I remember once reading in a text-book of organic chemistry: "The nutritional requirements of man are completely satisfied by a properly balanced diet composed of proteins, fats, carbohydrates, salts and water." The next sentence went on to say that vitamins were also necessary "not because of their nutritive value but because they prevent the development of certain diseases".

It seems impossible at the present time to draw any line of demarcation between substances which enter into the building up of bulky and prominent tissue constituents (e.g. proteins) and those substances which enter into the building of special tissue constituents such as enzymes or enzyme systems. Anything which may be utilized in the food enters into nutrition regardless of what its function may be, whether to provide a skeleton as in bone, energy as carbohydrate, building stones for muscle proteins or whether it is used in minute amounts to build up some special hormone or a part of some complex enzyme system. The term "nutrilite" like the term "vitamin" is a convenient one for designating organic nutrients which are effective in minute amounts, but the distinction between an ordinary nutrient and a nutrilite is not, so far as we can see, a very fundamental one.

(5) INHIBITING SUBSTANCES AND CONTROLLED GROWTH

The fact that the growth of cells, tissues and organisms is always restricted, is well recognized and it must be that there is chemical machinery operative to control this growth. I have little to contribute to this problem except to point out the fact that among the yeast nutrilites the same substances which can promote growth under certain circumstances may inhibit growth under other conditions. A striking and simple case of this is that of β-alanine, which in very minute doses inhibits growth, promotes growth in larger (though still minute) concentrations.

In the course of the studies on pantothenic acid, in my laboratory, we have often observed what seemed to be highly toxic substances (apparently derived from pantothenic acid itself) which interfered with quantitative tests. These inhibiting substances were effective in doses as minute as the doses of pantothenic acid used, and much more minute than would be required of ordinary poisons. It may well be remarked that whereas such substances as barium ions and brucine are toxic to yeast, the toxicity is of such a low order compared with the potency of pantothenic acid, that even crude barium or brucine pantothenate can be tested directly on yeast without interference. Yet other inhibiting substances did interfere with the tests and sometimes seriously. Von Euler *et al.* (1935) have observed the presence of fermentation inhibiting substances in yeast. Rapp (1939) has called attention to the general importance of inhibiting substances and Thimann (1939) has reviewed the question of the relation of auxins to inhibition of plant growth.

It will perhaps appear speculative, yet it seems worth while, to suggest that the nutrilites which stimulate growth may in some cases be converted during growth into agents which inhibit and restrict growth. Such agents have not yet been seriously investigated.

(6) POSSIBLE RELATION TO ACTION OF POISONS AND CHEMOTHERAPY

We are often reminded when we consider the action of poisons that the phenomena concerned are *interactions* between the poison and the materials of the living cell. It appears certain that some of the nutrilites discussed in this review are "indispensable cogs" in the machinery of living matter. If this is so, may not the subtle and inexplicable effects of a poison be due in some cases to interaction with these indispensable parts of the machinery? May not the selective and specific behaviour of toxic and inhibitive substances toward different tissues and different organisms have as its basis peculiarities in metabolism which would be reflected in the enzyme systems which are operative?

It appears likely to me that a knowledge regarding the "finer elements" which make up the complex machinery of living matter in its various forms is fundamental to the understanding of the action of poisonous substances and might well help to explain what now seems so difficult to understand, how a substance such as hexyl resorcinol, for example, can be relatively so much more toxic to certain types of cells (bacteria) than others, and how phenol is selective in an opposite direction.

The basic information on which to base a *science* of chemotherapy is largely lacking, and the great progress in this field has largely been due to trial and error. The fact that experiments *in vivo* are so often different in outcome from those done *in vitro*, makes the study more difficult, yet it again seems certain that the "finer elements" which enter into the machinery of different types of living cells must be better understood.

IV. SUMMARY

While yeast studies have often pioneered in the field of biology, serious progress in determining the nature of "bios", said to be indispensable for the growth of yeast, did not become evident until after a probable relationship to the "vitamins" was established. Even then the progress was very slow, and much confusion existed, partly because of the failure to recognize the differences existing between various strains of *Saccharomyces cerevisiae*, partly because of diverse methods of experimental study and the fact that the responsible agents were present in extracts in infinitesimal amounts and were potent in concentrations much lower than could be anticipated.

Now, nearly forty years after the discovery of "bios", five important yeast nutrilites are known chemically, at least three of which are vitamins. The five are inositol, thiamin, biotin, pantothenic acid and pyridoxin (vitamin B_6). Of these all except biotin have been synthesized in the laboratory.

One strain of yeast is apparently incapable of producing pantothenic acid or of growing indefinitely in its absence. Otherwise it appears that the three yeasts which have been studied are all capable of synthesizing the nutrilites in question. The effects of the nutrilites (with exception noted) are due not to their absolute indispensability but to the fact that their biological synthesis may limit growth. Experiments indicate that when long growth periods are concerned, one nutrilite may, in a measure, replace another.

It is evident that there are still unrecognized yeast nutrilites. These too may prove of general importance in biology, and like the known nutrilites may have intimate relationships with enzyme systems, the phenomenon of controlled growth, and the action of poisons and chemotherapeutic agents.

say that vitamins were also necessary "not because of their nutritive value but because they prevent the development of certain diseases".

It seems impossible at the present time to draw any line of demarcation between substances which enter into the building up of bulky and prominent tissue constituents (e.g. proteins) and those substances which enter into the building of special tissue constituents such as enzymes or enzyme systems. Anything which may be utilized in the food enters into nutrition regardless of what its function may be, whether to provide a skeleton as in bone, energy as carbohydrate, building stones for muscle proteins or whether it is used in minute amounts to build up some special hormone or a part of some complex enzyme system. The term "nutrilite" like the term "vitamin" is a convenient one for designating organic nutrients which are effective in minute amounts, but the distinction between an ordinary nutrient and a nutrilite is not, so far as we can see, a very fundamental one.

(5) INHIBITING SUBSTANCES AND CONTROLLED GROWTH

The fact that the growth of cells, tissues and organisms is always restricted, is well recognized and it must be that there is chemical machinery operative to control this growth. I have little to contribute to this problem except to point out the fact that among the yeast nutrilites the same substances which can promote growth under certain circumstances may inhibit growth under other conditions. A striking and simple case of this is that of β-alanine, which in very minute doses inhibits growth, promotes growth in larger (though still minute) concentrations.

In the course of the studies on pantothenic acid, in my laboratory, we have often observed what seemed to be highly toxic substances (apparently derived from pantothenic acid itself) which interfered with quantitative tests. These inhibiting substances were effective in doses as minute as the doses of pantothenic acid used, and much more minute than would be required of ordinary poisons. It may well be remarked that whereas such substances as barium ions and brucine are toxic to yeast, the toxicity is of such a low order compared with the potency of pantothenic acid, that even crude barium or brucine pantothenate can be tested directly on yeast without interference. Yet other inhibiting substances did interfere with the tests and sometimes seriously. Von Euler *et al.* (1935) have observed the presence of fermentation inhibiting substances in yeast. Rapp (1939) has called attention to the general importance of inhibiting substances and Thimann (1939) has reviewed the question of the relation of auxins to inhibition of plant growth.

It will perhaps appear speculative, yet it seems worth while, to suggest that the nutrilites which stimulate growth may in some cases be converted during growth into agents which inhibit and restrict growth. Such agents have not yet been seriously investigated.

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THE INTERPRETATION OF LEAF AND ROOT IN THE ANGIOSPERMS

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I. INTRODUCTION

SELF-MAINTENANCE has been recognized since the days of Aristotle as a fundamental character of living things, but it was Spinoza in the seventeenth century whose insight first gave this idea its full significance, when he formulated the proposition: "The effort by which each thing endeavours to persevere in its own being is nothing but the actual essence of the thing itself."[1] He thus set the principle in the forefront of biology, by making the urge towards self-maintenance, not merely a character of living things, but the very gist of life. Variants on the same conception have been recorded repeatedly by more recent writers, though they have seldom, if ever, faced its implications as resolutely as did Spinoza. Rignano, in 1929, for instance, gave it partial expression, when he wrote of "la tendance qu'a tout organisme à conserver, sans variation, son propre état physiologique stationnaire, ou à y retourner chaque fois qu'il vient à être troublé". Bertalanffy, again, in 1933, crystallized one aspect of Spinoza's dictum into "the law of biological maintenance". This urge to self-continuance is revealed in different ways in different living creatures; in the flowering plants, with their power of indefinite growth, it takes a special form—that of repetition. This repetitive branching has been well described as "die wahre morphologische Allgemeinheit der Pflanze" (Schultz-Schultzenstein, 1861). The whole plant may be said to consist of a series of shoot generations, together with a series of root generations; every individual lateral branch is a repetition, modified in varying degrees, of the original primary shoot, while every lateral root similarly repeats the primary root. The plant "endeavours to persevere in its own being", and this perseverance expresses itself in repeating that being time after time, each daughter shoot or root becoming in turn a parent shoot or root. In other words, since the capacity for branching makes each individual shoot or root potentially a complex, the whole becomes "eine Gesellschaft unter sich verwachsener älterer und jüngerer Individuen" (Schultz-Schultzenstein, 1861). The fact that even the most elaborate plant system proves to be completely amenable to analysis when treated as a root-and-shoot complex, suggests that these two elements may be, in some sort, primary units. In a former paper (Arber, 1930) arguments have been brought forward in support of this concept, and the conclusion has been reached that, as a morphological category, the *shoot* has a higher degree of validity than *stem* and *leaf*, which are often opposed to it as representing basic elements in plant construction. It is not necessary here to reiterate the arguments on which this conclusion was founded, nor those which led to the rejection of another view, the *phyton* theory. It may, however, be recalled that stress was laid upon the illogical results which follow from the phyton theory, and, on the other hand, upon the obstacles to delimiting leaf and stem externally or anatomically, which make it impossible to differentiate these categories with precision. More recently Foster (1936), as a result of his detailed ontogenetic studies, has come to a conclusion which harmonizes with that just expressed about the leaf and the stem. He writes: "It seems clear that any effort to isolate the leaf or stem, at least from a morphogenetic standpoint, is unsupported by the facts of development."

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[1] "Conatus, quo unaquaeque res in suo esse perseverare conatur, nihil est praeter ipsius rei actualem essentiam". [Spinoza, B. de] "B. D. S." (1677), *Ethices*, Pars. III, Prop. VII, p. 102. For translation, Spinoza, B. de (1930), Prop. VII, p. 114.

The notion that the shoot, rather than the stem or leaf, should be taken as a unit, is, indeed, far from being a new one. Sachs, though his concern with morphological categories was only incidental, recognized the essentials of the problem with characteristic acumen. In the words of his translators (1875), "*The morphological conceptions of Stem and Leaf are correlative*; one cannot be conceived without the other...the expressions Stem and Leaf denote only certain relationships of the parts of a whole—the Shoot."

When we are thinking comparatively of the various well-attested modes of analysing the plant body, it must always be borne in mind that none of them can be "right" or "wrong" in any absolute sense. Each way of contemplating the plant has its own individual value, and, though one way may be preferred because it appears to be less inadequate than the others, yet the rejected ways—though we have turned aside from them as relatively unsatisfying—may often lead to some element of truth, which has slipped out of sight in the accepted view. For example, the idea that "leaf" and "stem" are fundamental units of the plant body, though it does not, in general, accord so well with the facts as the "shoot" hypothesis, yet has the merit of stressing the idiosyncrasies of the leaf, which are apt to receive less than their share of notice when the shoot is treated as the basic category. What is needed is a theory which, while retaining the shoot as a primary unit, will yet incorporate a full recognition of the tendencies which individualize the leaf; such a view should include what is valuable in the contrasting outlook, which is based upon the antithesis of stem and leaf. The difficulty of the problem has been realized since early times. After the lapse of more than three hundred years, we are still obliged to echo Adrian Spieghel's words, written in 1606: "But what is...the leaf? Long and oft have I sought an answer to this question, without finding anything with which I could satisfy you and myself."[1]

If we arrive at some interpretation of the leaf in relation to the shoot, we have still to face the further problem of how the root is to be regarded. It becomes a question whether we shall remain content to treat root and shoot simply as parallel and incommensurable, or whether it may be possible to reach some unified idea for the plant body, which will include them both.

It is the double problem shadowed out in this Introduction which forms the subject of the present essay. Firstly, and mainly, we shall consider how to develop the hypothesis of the shoot as a unit, on lines that will include an adequate picture of the relation of leaf and shoot; and secondly, we shall see whether any standpoint can be gained, from which the root can be visualized in relation to the shoot, rather than in antithesis to it.

II. THE INTERPRETATION OF THE LEAF IN RELATION TO THE SHOOT

There is little doubt that the principal factor in the development of modern morphological botany is the work of Troll and his school, which is being summarized in his encyclopaedic *Vergleichende Morphologie der höheren Pflanzen*, which has been in course of publication since 1935. Troll received his training in the Munich laboratory, and his series of volumes to some extent covers the same ground as Goebel's *Organographie* (ed. 3, 1928–33), though planned on a more generous scale. It is indeed a colossal undertaking, for the account of the vegetative organs, which is not yet completed, already covers more than 2000 pages. Troll's conspectus differs from Goebel's work in its greater homogeneity, which depends upon the fact that he has gone back to Goethe as his guide, and has reinstated the type concept, for which the poet was responsible, and has employed it consistently throughout. The type idea is undoubtedly of great value in evoking order out of a plexus of heterogeneous details, but it has, on the other hand, serious drawbacks. This is not the place for a general criticism of it; an attempt has been made in a former paper in this journal (Arber, 1937*a*) to modify it by replacing the notion of the *type* by that of *parallelism*. One of the defects of typology is that it involves the temptation to carry analysis unduly far, in order to secure units of the greatest possible simplicity to serve as types The shoot, for instance, when treated as a unit, is liable to prove too complex for facile thought of the text-book kind; if, however, appendages and axis are imagined in artificial detachment, they become more easily amenable to mental handling, though, unfortunately, this simplicity is gained at the expense of realism. The fact that Goethe was not burdened by specialist knowledge of botany was of some advantage in enabling him to take a bold and comprehensive view, but it had the disadvantage that it left him unguarded against this tendency to oversimplification, which led him to concentrate on the leaf, as an individual object, in abstraction from the shoot of which it forms a part. Troll (1937) adopts the same attitude, and adheres to the related Wolff-Braun theory that "das Blatt ein weiter nicht ableitbares *Grundorgan* ist". The idea that the leaf is an ultimate "given" element in the construction of the plant body, and that root, stem, and leaf, form three fundamental categories to which members of the plant can be assigned, is the basis of Troll's morphology, and it is accepted by other modern writers. Uittien, for instance, wrote in 1939: "La feuille est une unité morphologique, c'est là une des 'idées' de la plante."

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[1] Spieghel, A. (1606), p. 7: "Sed quid...Folium? Hoc diù multùmq́. quaesivi, nec quidquam inveni, quo mihi vobisq; satisfaciam."

Though a century and a half has passed since Goethe's epoch-making essay was published, it is true that many of his botanical notions even to-day retain the vitality impressed on them by his vigorous mind; but it is equally true that their value lies in supplying stimulus to present-day thinking, rather than in offering a ready-made system to be adopted as it stands. It is indeed regrettable that his enthusiastic adherence to the ideas of Goethe and his followers has led Troll to the point of accepting root, stem, and leaf, as units which are not susceptible of further analysis;[1] for this view acts as an inhibiting agent, sterilizing potential lines of thought which are often distinctly implicit in his work. If one departs from his standpoint so far as to treat the leaf as something which one may try to interpret, instead of as something which is an ultimate datum of experience, his *Vergleichende Morphologie* proves an unparalleled source book. Its exact and beautifully illustrated descriptions, though designed to demonstrate developments arising out of Goethe's leaf concept, become far more illuminating when the question of the relation of the leaf to the rest of the plant is not put "out of bounds" as it is by Troll.

In 1790, the very year in which Goethe first expressed his ideas about the leaf, a hint at a different way of looking at the matter came from the philosopher, Immanuel Kant—a hint which, under the sway of Goethe's influence, seems to have fallen on stony ground. Kant suggested that "each branch or leaf of a tree may be regarded as merely engrafted or inoculated upon it, and therefore as a tree with an existence of its own, simply attached to another, from which it nourishes itself".[2] This idea of leaf and lateral shoot as parallel members, each with its own individuality, and with a certain equivalence to the parent plant, has greater suggestive force for the modern morphologist than Goethe's brilliant but limited scheme.

Despite the wide acceptance of Goethe's views in the nineteenth century, there were botanists who did not express their ideas in terms of the leaf concept, but maintained opinions which were more akin to those of Kant. Oken, for instance, whose extravagant "nature philosophy" is illumined here and there with flashes of genuine insight, wrote in 1810 that "A leaf is a whole plant".[3] Half a century later, Schultz-Schultzenstein (1861) published a paper in which he laid his finger on the weak spot in the theory of Goethe and his followers—the fact that both "leaf" and "stem", considered as "types", are merely vague abstractions. He realized that it was a retrograde step to adopt the leaf as an ultimate unit, and that what was wanted was a recognition of the fact that the nature of the leaf was in itself a problem—"das grösste Räthsel der Botanik"—for which a solution ought to be sought. Unfortunately he did not see that the defects of stem and leaf as morphological units might be overcome by synthesizing them into the shoot concept. The result was that, though he made a penetrating comparison between a compound-pinnate leaf with its rachis and pinnae, and a stem bearing leaves, he lost himself in confused notions about the "anaphyte", and failed to amalgamate his disjointed ideas into any consistent theory of the leaf.

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[1] "Grundform ist das Blatt aber auch insofern, als es einer Ableitung aus anderen Teilen des Vegetationskörpers nicht fähig...ist." (Troll, 1938, p. 957.)

[2] "Daher kann man auch an denselben Baume jeden Zweig oder Blatt als bloss auf diesem gepropft oder oculirt, mithin als einen für sich selbst bestehenden Baum, der sich nur an einen andern anhängt und parasitisch nährt ansehen." (Kant (1790), from 1908 edition; see also Kant (1928), p. 19.)

[3] "Ein Blatt ist eine ganze Pflanze mit allen Systemen und Formationen." (Oken, 1810, **2**, 72, § 1144.)

Seven years later Casimir de Candolle (1868) made an advance of permanent value when he described a peltate or cylindrical leaf as a branch with its "cône terminal" atrophied at the summit, while he interpreted the ordinary bifacial leaf as a branch in which the "cône" has atrophied on the posterior (ventral) face also. The suggestion that the leaf was probably a modified branch had already been thrown out by Christopher Dresser (1859), but, unlike de Candolle, he did not offer any scientific study to justify his opinion.

We see thus that, on the subject of the nature of the leaf, botanists have been divided into two opposed camps—the disciples of Goethe, who accept this member as an organ *sui generis*, which defies further analysis, and those who have developed the view foreshadowed by Kant, according to which leaf and shoot have a certain equivalence. As a preliminary to getting more closely to grips with the problem—which becomes essentially a question of the relation of shoot and leaf—it is necessary briefly to review the features in which a typical leaf differs from a typical shoot. This involves a reiteration of facts which are obvious to the point of triteness, but which demand restatement because their significance has not been appreciated to the full. For simplicity, attention may be focused upon the contrasts between an ordinary foliage leaf and a lateral shoot axillary to it.

At a vegetating shoot apex, a leaf arises as an upgrowth, the base of which coincides with part (or the whole) of the circumference of the axis. Though this base may enclose the growing apex, it does not normally incorporate it. The leaf rudiment is, as it were, an arc of tissue freed from the axis just below the shoot tip, and its prime characteristic is that it has an outer (lower) face which is continuous with the outer face of the axis, and an inner (upper) face, which is turned towards the growing apex. These two faces intersect in a margin which runs, from the lateral edges of the leaf insertion, up on either side to the tip of the leaf. In emphasizing the essential dorsiventrality of the structure, and the importance of the region of intersection of the surfaces, Bower's analysis (1916) is of special value. "The leaf", he wrote, "is a bifacial member with margins defined structurally, though these may be obscured." The picture which the word "leaf" raises in one's mind is primarily that of a flat expansion of green assimilating tissue, and it is from the margins at the intersection of the upper and lower surfaces of the rudiment that this expansion is, as a rule, produced.

When we turn to the lateral shoot axillary to the leaf which we have been considering, we find that its most noticeable difference from the leaf is that it is not a two-faced member. Its symmetry resembles that of the parent shoot in being radial. Its whole surface corresponds with the outer surface of the parent stem, and there is thus nothing in its construction equivalent to the inner (upper) surface of the leaf.

The other main difference between the leaf and its axillary shoot lies in the fact that the apical growth of the leaf lasts, as a rule, for a short time only, while that of the shoot is much more prolonged. Certain characteristics of the leaf are probably, as we shall see later, directly attributable to this early cancellation of terminal growth in favour of basal intercalary growth.

An account of the typical leaf which brings it into relation with the shoot, while recognizing in some degree the two points of difference to which we have just alluded, may be found in Casimir de Candolle's theory. We may re-express the view which he advocated by saying that *the leaf is a partial-shoot, arising laterally from a parent whole-shoot*. Why the leaf should show this partially inhibited development, while the adjacent lateral shoot-bud possesses that completeness which the leaf characteristically lacks, is one of the mysteries of ontogeny. A factor among those responsible for this contrast may perhaps be that the leaf is initiated by differential growth in a region which is wholly meristematic, whereas the lateral shoot-bud originates slightly later, and is thus in an earlier meristematic phase than the surrounding parts. In respect to this time element, the shoot-bud's relation to its surroundings is to some extent comparable with that of an embryo, which is always younger than its environment, while the close contemporaneity of the most recently formed leaf with the shoot apex from which it arises, makes the leaf, in a completer sense than the lateral branch, an integral part of the shoot. Modern work on ontogeny indicates that the fate of the lateral members is settled at a very early period of their life, and it is probable, as Eichler suggested in 1875, that the first stages of development which come within our observation, are not by any means the earliest, and that "wenn die Theile als Zellhügel unterscheidbar werden, schon viele Veränderungen vor sich gegangen sein können". If this be so, the influences that decide the leaf or shoot nature of an outgrowth must be exercised, as it were, pre-natally. Casimir de Candolle's theory throws no light on the nature of these influences, but it does, at least, present a coherent account of certain features characterizing an ordinary bifacial foliage leaf of a simple type. His partial-shoot idea is not, however, as it stands, adequate even for the complete description of this ordinary leaf, and still less for the understanding of why there should be so wide a range of other variants, such as the unifacial, peltate, compound-pinnate, etc. When we ask in what direction we can look for help in increasing the scope of de Candolle's theory, the answer is that the clue lies in the application of Spinoza's self-maintenance proposition, the morphological aspect of which has been slightly touched upon in the Introduction. When Spinoza wrote of each thing endeavouring to persist in its own being, no doubt he was thinking of the organism as a whole, but his doctrine applies with equal force to the individual members making up the plant body. Moreover, "the endeavour to persevere in its being" means more than may be recognized at first glance. It implies the endeavour to bring the "*determinata natura*" of that being into a condition of completeness or perfection in its own kind. When we apply these ideas to the problem of the leaf, we see that they suggest the hypothesis that this member may be interpreted as *a partial-shoot which has an inherent urge towards the development of whole-shoot characters*. This view has no phylogenetic implications; it does not commit us to any opinion as to the origin of the leaf as a matter of history, but is concerned with what the leaf actually *is*, here and now. There is no need to suppose that the leaf passed ancestrally through a complete-shoot phase, and was subsequently reduced to its present incomplete form. Being essentially dorsiventral, it is part of its very nature never to have been at any stage more than a partial-shoot. This point needs stressing because the nineteenth-century trick of thought, which was prone to give a phylogenetic twist to morphology, is a danger to the unwary even to-day. It is based on the false assumption that any statement about the morphological equivalence of an organ will remain valid if re-expressed in historical terms. The error in assumptions of this type is more obvious when individual development rather than phylogenetic history is being considered, since here we know the past, instead of merely guessing at it. For instance we may rightly conclude from its situation, form, and nervation, that a certain calyx segment is equivalent to two sepals, and yet we may also know, from the observed facts of development, that we should be wrong if we assumed that this double member had originated from two rudiments which subsequently fused.

Part of the preceding discussion, as well as the formulation of the partial-shoot hypothesis, will probably be criticized adversely on the ground that the use of such words as "urge", "endeavour", "effort", and "perseverance", seems to attribute a human psychology to the plant. The employment of these words is, however, unavoidable, because we have no other set of terms in which to express that compulsiveness by which the plant works towards a certain end, which, if we were concerned with a self-conscious organism knowingly pursuing a train of activity, would be recognized as a goal. For this reason, these ideas, and the words needed to express them, will not be avoided in the present essay; but it must be understood that they are not to be taken in the crudely anthropomorphic sense. A critique by Kozo-Poljanski (1936) emphasizes the fact that modern morphology has outstripped "the familiar alternatives". To the difficulties surrounding the "third" conception which he discusses, may be added that of embodying thought for which our stereotyped language no longer suffices.

Up to this point we have dealt with the problem of the leaf in somewhat generalized terms; but in order to test the validity of the interpretation here offered, we must now turn to a more detailed comparison of leaf and shoot. We may make this comparison under two headings, taking firstly the evidence that leads us to recognize the leaf as a shoot, though an imperfect one; and secondly, the evidence that indicates that this partial-shoot has a definite trend towards self-completion as a whole-shoot. The separation of these two classes of evidence is somewhat artificial; but there is a certain convenience of presentation in keeping them apart.

III. THE LEAF AS A PARTIAL-SHOOT

Before considering the grounds for the partial-shoot hypothesis, it may be well, in the first place, to enquire whether there is anything in the development of shoot and leaf which should, at the outset, preclude this view. The answer from the literature is a negative one, since there seems to be no recorded difference of a fundamental kind between the ontogeny of the two. The shoot grows by means of an external *tunica* and an internal *corpus*, while the leaf is produced either by *tunica* alone, or by *tunica* and *corpus* jointly (Schmidt, 1924). As we shall see a little later, there are also certain additional resemblances in the detailed development of shoot and leaf.

The feature in leaf ontogeny, which is ultimately responsible for many of the differences between shoot and leaf, is the early failure of the capacity for terminal growth. At their initiation leaves grow apically, and the tip is often relatively large in early stages, but the meristematic activity of the apex soon comes to an end, and the focus of cell division is transferred to the lower regions of the leaf. In *Nicotiana Tabacum* L., for instance, apical growth ceases, and is replaced by basal growth, when the primordium is 2–3 mm. in length (Avery, 1933). We may describe the early development of the leaf as a temporary effort after whole-shoot character—an effort which soon dies down. The inhibited apical region may often be recognizable as a point at the tip of the mature leaf (e.g. *Cucurbita Pepo* L., Troll, 1938). It is analogous to the residual apices of certain shoots (e.g. *Uvularia*, Arber, 1925, Fig. xxxv, p. 58). The subsequent elongation of the leaf in its lower regions can be compared with the basal intercalary growth of internodes which occurs in many shoots, for instance, in the haulms of the Gramineae. It may be suggested that not only basal elongation, but also lamina development, is to some extent correlated, as a process of compensatory growth, with the limitation of apical extension. Moreover, this and other structural limitations may possibly also be connected both with the ephemeral character of the leaf, and with its precocity. Leaf fall is a much more usual phenomenon than branch fall, though this sometimes occurs, as in the natural pruning of certain poplars. At the growing point of any shoot, the leaf rudiment as a rule soon overtops the shoot apex, which may yet continue to grow and develop long after the leaf, which is its offspring, has aged and fallen. It seems indeed to be generally true for living things that precocity and a capacity for prolonged and full development are seldom found together. So it is not, perhaps, too speculative a suggestion that the early maturity and short life of the leaf may be related symptoms of its imperfect character as a partial-shoot.

When we turn from the question of apical growth to the lamina development which is the most characteristic feature of foliage leaves, we find that in dicotyledons this blade formation is predominantly pleuroplastic, i.e. arising from the margins of the leaf rudiment. This feature is shown in Trécul's figures in 1853, while Prantl (1883) and later writers have appraised it more exactly. Avery's modern description (1933) of the development of the tobacco leaf (*Nicotiana Tabacum* L.), to which we have already referred, furnishes a good example of this type. He states that the leaf is little more than an embryonic midrib until it reaches 0·6 mm., or more, in length. The lamina then makes its first appearance as two ridge-like projections initiated by the activity of a row of sub-epidermal cells forming a marginal meristem, which extends along either side of the midrib primordium. Pleuroplastic development may also be responsible for the growth of wings from the leaf base, e.g. the sheath wings of *Aesculus Hippocastanum* L. (Foster, 1929). The pleuroplastic process seems at first glance very different from the ontogeny of a shoot, but Schüepp (1938) has pointed out that in the shoot apex the superficial layers grow as a lamellar meristem, and thus produce folds which form the leaves. It follows that the leaf lamina grows from the peg-like rudiment of the leaf in much the same manner as the leaves themselves grow from the shoot axis. The continuous spiral path of leaf development in the shoot is represented in the leaf by the simpler path of lamina development, which in the pleuroplastic leaf follows the junction line between the upper and lower surfaces.

These growth comparisons open the way to an analysis of the leaf itself, considered as a shoot "in little". The elements of the leaf which may be regarded as entities parallel to the axial or stem elements of the shoot, are: the median region of the leaf-base; the petiole; the midrib of the simple leaf; the rachis, and racheoles, of the compound leaf; and the non-winged leaf-apex. The elements of the leaf, on the other hand, which parallel the foliar elements of the shoot are: the stipules; the lateral wings of the sheathing leaf-base; the lamina of the simple leaf; and the laminae of pinnae of various orders of the compound leaf. One item in this correspondence—that between petioles and shoot axes—may seem to be partially vitiated by the delayed development of the petiole, which often appears so late as to suggest a secondary origin. This delayed petiole production should be held, however, to correspond to the belated development of internodes in shoot buds, which may show no elongation even at a stage at which the bud has produced a large number of leaves.

We have spoken of the stipules as related to the petiole in the same way as the leaves to the shoot axis; this comparison may now be carried rather further. The relation of stipules to the leaf of which they form a part may be compared with the relation of cotyledons to the primary shoot, or of prophylls, or bracteoles, to a lateral shoot. Stipules often differ in form, size, texture, etc. from the succeeding members of the leaf, while cotyledons and prophylls are distinguished from the succeeding leaves by differences of a corresponding kind. In their paired character, also, lateral stipules recall the seed-leaves, prophylls, or bracteoles of dicotyledons. Axillary stipules, on the other hand, show some resemblance to the single seed-leaf, prophyll, or bracteole, of monocotyledons, which often has a duplex type of anatomy. Although among stipules the members of each pair are commonly alike, there are examples in which they differ, e.g. certain members of Rhamnaceae and Sterculiaceae (Glück, 1919). Correspondingly, though paired prophylls are nearly always alike, in certain cases, e.g. *Geranium Robertianum* L., they differ in size (Goebel, 1931). Stipules tend to be asymmetrical with reduction of the lamina on the side away from the leaf-base, and here again a corresponding asymmetry recurs in prophylls (Troll, 1935, 1937).

Stipules as a rule differ from prophylls and cotyledons in one important respect —namely that they subtend no axillary buds. There are, however, rare exceptions (certain Alchemillas, Bohlin (1899), and *Thermopsis lanceolata* B.Br., Velenovský (1910)), so that the difference in this respect between stipules, on the one hand, and cotyledons and prophylls, on the other, is not absolute.

It is not only true that shoot and leaf may be considered in serial relation—the leaf being regarded as a reduced offspring which partially repeats the characters of the shoot—but also that the leaf may occasionally bear outgrowths which are related to the leaf, as is the leaf itself to the parent shoot. For instance, the relation of an enation to the foliage leaf which bears it, or of the nectary scale of *Ranunculus* to the honey-leaf (Arber, 1936), or of the corona of *Narcissus* to the perianth (Arber, 1937*b*), may be compared with the relation of a leaf to its parent axis. That there is indeed a basic similarity between these relations is indicated by the anatomy; we find that leaf-enation, honey-gland, and corona, all have the xylem faces of their bundles turned towards the parent member. A similar relation holds good also in certain anomalous shoots, such as the phylloclade of *Ruscus Hypoglossum* L. This structure may be interpreted as a shoot in which the reduced axis has had its individuality entirely absorbed by its own prophyll, so that the whole member appears to be a single leaf. Some of the phylloclades are flower-bearing, and in these a bract arises from the surface of the phylloclade, with the inflorescence in its axil. This bract may occur either on the upper or under surface, but, on whichever face it grows, it has its xylem directed towards the parent phylloclade (Arber, 1924, Fig. 21H, I, and Fig. 22B, C, p. 238). We see the same principle at work in another anomalous type of leaf—the spikelet-bearing lemma of "Nepaul barley", *Hordeum trifurcatum* Jacq. Here the lemma (flowering glume, bract) has a normal spikelet in its axil, but from its own upper surface, near its apex, it produces other accessory spikelets. There is not actually any leaf-borne bract to correspond with that of *Ruscus Hypoglossum*, but each of the accessory spikelets is orientated as if it arose in the axil of a bract with its xylem facing the parent lemma (Arber, 1929*b*, Fig. 5, pp. 520, 526–7). In *Nasturtium officinale* R.Br., again, one sometimes finds an accessory flower set within each petal, and drawing its vascular supply from it. The vascular system of the pedicel of the accessory flower is so orientated as to show that, if an axillant bract had been present, it would, as in the case of Nepaul barley, have been related to the petal as to its parent axis (Arber, 1931*a*, p. 192, and Fig. 10, B_2 and B_3, p. 190).

It is not only in its external form, and in its relation to members of the next generation, that the leaf may be compared to the shoot; the resemblance extends also to the nervation. Uittien (1928) has shown that leaves tend to be pinnately nerved or divided when the main axis of the shoot in the same species exceeds the lateral axes in length; and palmately nerved or divided, when the lateral axes exceed the main axis. Sachs (1875), also, used the words "racemose" and "cymose" for types of leaf venation, so he evidently realized the parallelism of leaf and shoot in this respect. He had not, however, reached Uittien's generalization, though Oken, many years before, had given a sign that he had faintly foreshadowed it. He wrote:

"From the arrangement of the leaf nerves one can deduce the structure of the whole plant and determine its character. The leaf is the table of contents of the stem."[1]

In thinking about the resemblance of shoot and leaf, we must not, however, belittle the obvious difference that the leaf is usually extended in one plane only, while the shoot is radially symmetrical. Oken was fully alive to this point; in his words, "The leaf is a tree of special form, the branches or fibrous bundles of which all lie in one plane".[2] This difference is not, however, absolute. The tendency to radiality and the tendency to dorsiventrality coexist both in shoot and leaf, but the relative emphasis on these tendencies differs in the two—the shoot being predominantly radial, but with an underlying trend towards dorsiventrality, and the leaf being predominantly dorsiventral, but with an underlying trend towards radiality. These underlying tendencies often come to the surface. The lateral shoot, for instance, frequently inclines to dorsiventrality in its attachment and prophyllar regions. A lateral branch cannot, indeed, but have a certain dorsiventrality from the start, since a parent shoot does not normally give rise to a daughter shoot except from the side, so that a lateral shoot has an asymmetrical relation with its parent. In anatomy, also, the base of the lateral shoot is by no means completely radial. A not uncommon form of anatomical connexion between a lateral shoot and its parent shoot and axillant leaf is the division of a shoot bundle into three parts, the median member serving the leaf, while the two wing members of this triad division approach one another on the inner side of the leaf bundle, thus forming a bilaterally symmetrical group for the lateral shoot—de Bary's "bundles of insertion" (Bary, 1884; Arber, 1932, p. 170, footnote). These paired strands have their xylems facing one another, so, by their division, a ring is easily produced. The leaf, on the other hand, since it is served by an arc or a single bundle, with xylem above and ploem below, is committed, at any rate at its start, to a dorsiventral scheme of anatomy. For, from such an arc or single bundle, simple division of the customary type will inevitably produce a complex of bundles all lying with xylem above and phloem below. The contrast between this type of structure and that of the branch system is not, however, always complete, for it sometimes happens that the paired bundles of the bud-base do not branch in such a way as to radialize the anatomy perfectly; in the pedicels of the Cruciferae, for instance, there is often reduction of vascular tissue on the upper side, so that the structure shows a hint of dorsiventrality akin to that of the leaf (Arber, 1931*a*, Fig. 8A1, p. 186). Moreover, the tendency to dorsiventrality in the bases of lateral shoots may even reveal itself in the external form. Troll (1935) figures, for instance, a branch of a species of *Dracaena*, enclosing more than half the circumference of the parent axis, thus recalling a leaf with a broad sheathing leaf-base.

The dorsiventrality of lateral shoots is not always confined to the base. In the elm (*Ulmus*), for instance, the primary seedling shoot is radially symmetrical, but all the laterals, on the other hand, are dorsiventral and distichous (Schoute, 1937). *Coriaria myrtifolia* L. is a more extreme case. Here the lateral shoot systems closely resemble compound-pinnate leaves, but this tendency to dorsiventrality becomes predominant only in the later stages of development. In the bud the shoot is found to be radially symmetrical; the leaves are eventually brought into one plane by torsion of the internodes and curvature of the leaf bases.

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[1] "Aus der Anordnung der Blattrippen kann man daher den Bau der ganzen Pflanze erkennen, und ihren Charakter bestimmen. Das Blatt ist die Anhaltsanzeige des Stammes." (Oken, 1810, **2**, 72, § 1146; for a translation, see Oken, 1847.)

[2] "Das Blatt ist ein Baum von besonderer Form, ein Baum dessen Aeste oder Faserbündel alle in eine Ebene liegen." (Oken, 1810, **2**, 72, § 1144.)

IV. THE LEAF AS A PARTIAL-SHOOT, WITH AN URGE TO WHOLE-SHOOT CHARACTERS

The question whether the leaf has in reality, as is suggested by our present hypothesis, an urge towards whole-shoot characters, is a problem that cannot be considered in isolation. The likelihood that this urge has a real existence will be increased if it can be shown that there is, throughout the shoot complex, a tendency for the shoot of each generation to compete with, and attempt to replace, that of the previous generation. The urge of the leaf to reach the status of the shoot which is its parent, and even to replace it, would then be merely one link in a chain of tendency recognized throughout the plant. It seems demonstrable that a recurrent trend of this kind does, in fact, exist, for the subordination of parent shoots to lateral shoots is an extremely common phenomenon; it is the conspicuous feature of all sympodial and cymose branching. As examples we may cite the lilac (*Syringa vulgaris* L.), in which the terminal bud dies during summer and is replaced by two lateral shoots, from the axils of the pair of leaves below it; and the lime (*Tilia cordata* Mill.), in which a single lateral shoot each year replaces the terminal bud, which itself dies away. The details of these instances are well illustrated by Troll (1935). These sympodial developments may be associated, as Troll has suggested, with acrotony; when the lateral buds become progressively stronger as the end of the shoot is approached, the uppermost are the more likely to replace the terminal bud. An example of a different kind, also showing the laterals dominating the main shoot, is the winter bud of such a plant as *Potamogeton crispus* L. (Arber, 1920, Fig. 42, p. 67), in which the horny turion exists only to serve as a vehicle for its own lateral buds, which grow out in the spring. A number of cases are known in which flowers are actually terminal to inflorescences or partial inflorescences—that is to say, in which a lateral shoot (flower) replaces the apex of the parent shoot (inflorescence). Examples are found among the Gramineae. In *Streptochaeta* (Arber, 1929*a*, p. 43); *Luziola Spruceana* Benth. (Arber, 1928, p. 402); and *Anthoxanthum* (Arber, 1927, p. 475), the spikelet apex is replaced by a single flower; while in *Lygeum Spartum* Loefl. (Arber, 1928, pp. 403, 404) it may be replaced by two flowers back to back, with their bracteoles fused at the base, and no trace between them of any residual apex belonging to the spikelet. The process of replacement of the growing apex of the main shoot by its laterals may go so far that the uppermost laterals receive not only their normal supply of vascular tissue, but also any vascular tissue left in the shoot, so that the shoot apex, if it survives, is non-vascular, e.g. certain Fumarioideae (Arber, 1931*b*, pp. 340–2; and see Addendum).

Just as a lateral shoot may dominate or even incorporate the apex of the parent shoot, so a leaf may dominate or even incorporate the apex of the shoot of which it is part. The phylloclade of certain Liliaceae—*Danae*, *Ruscus*, and *Semele* (Arber, 1924, especially pp. 250–1)—which has already been mentioned in another connexion, is a short shoot reduced essentially to its first leaf (prophyll), which appears actually to incorporate the apex of the dwarf shoot to which it belongs. A phylloclade may terminate a long shoot, and it is then an example both of a lateral shoot replacing the apex of a main shoot, and of a prophyll over-mastering the secondary shoot of which it is the only leaf member. In the "droppers" of *Tulipa* and *Erythronium*, and the "sinkers" of *Orchis* (Sharman, 1939)—foliar structures in which buds are carried down into the soil away from the parent axis—we have other examples in which the leaf dominates the situation, incorporating the shoot into itself. If Hagerup's theory (1930) is well-founded, the tendril in the Passifloraceae, as well as the one-armed tendril in the Cucurbitaceae, is to be interpreted as a thread-like branch with a pseudo-terminal leaf. Here it is the prophyll of a short shoot which becomes tendrillar, and is the most conspicuous feature of the shoot.

The attempt of the leaf to dominate the parent shoot is revealed even in early ontogeny. It has been shown that some influence from the leaves has an inhibiting effect upon both axillary and terminal buds, causing them after a time to stop elongating and to turn into winter buds (cf. Snow, 1929, and references there given).

The subordination of the shoot apex to its own lateral shoots, or to its own leaves, may be paralleled within the leaf itself, in the domination of the leaf apex by lateral leaflets. The median region of a simple leaf, or the terminal pinna of a compound leaf, may be considered as the primary part of the leaf, since it is served by the midrib, whereas the rest of the leaf is served by lateral branches from the midrib. In some pinnate leaves this end pinna may be reduced or absent; in *Biophytum sensitivum* DC., for instance, it is described as an insignificant thorny process (Troll, 1939). In *Bauhinia yunnanensis* Franch. there are only two lateral pinnae, and here the terminal pinna is reduced to a mere point, while in *Amicia Zygomeris* DC. it is absent. The process of inhibition of the primary part of the leaf may even go so far that certain apparently simple leaves may be interpreted as consisting of two lateral pinnae in a state of fusion, the median pinna being absent, e.g. *Cercis Siliquastrum* L. (Fries, 1909). A corresponding process may occur in leaves belonging to other groups. Troll (1939) has figured a series from the Berberidaceae, passing from *Achlys triphylla* DC., with its ternate leaf, through *Jeffersonia binata* Bart., with the median lobe indicated only by a point, to the primary leaf of *Podophyllum peltatum* L., in which the median lobe is entirely absent. He also exhibits a series among species of *Passiflora*, in which there are leaves with three equal lobes, and also such leaves as those of *P. capsularis* L., in which the median lobe is merely a point. The same median reduction may occur in the individual members of a compound leaf; in *Oxalis Ortgiesi* Regel, for instance, the pinnae of the ternate leaf recall in their form the whole leaf of *Passiflora capsularis*.

We have now followed in several fields the tendency of lateral structures to aspire, as it were, to take up the role of the parent, for which they are understudies. We have seen this tendency in the relation of a shoot of one order to the shoot of the previous order; in the relation of a leaf to the shoot which bears it; in the relation of branches (pinnae) of the leaf to the terminal pinna; in the relation of the lateral lobes of a simple leaf to the median region; and in the relation of the lateral lobes of the pinna of a compound leaf to the median part of the pinna. When we study the nervation of the leaf, rather than its external form, we again find the same principle coming into play; as we have already noticed, Uittien (1928) has shown that there is an actual correlation between the subordination of the main shoot to lateral shoots, and the subordination of the leaf midrib to lateral veins; that is to say, when the shoot branching is "cymose", there is a tendency for the leaves to be palmately nerved. Palmate venation may be visualized as a type in which the main vein is shortened by suppression of its earlier "internodes"; the laterals are thus more important relatively to the midrib than in pinnate leaves, in which the midrib is elongated and dominates its own branches. Another more obvious example of laterals exceeding the midrib in importance is found in the anatomy of *Acacia* phyllodes and various Irid leaves, in which the main laterals jointly form a pseudo-midrib (Arber, 1921, Figs. 40–44, etc., p. 318). Predominance of laterals also occurs in both dorsiventral and ensiform species of *Moraea*, in which the median region is supplied by veins which are smaller than the laterals (Arber, 1921, Figs. 36–8, p. 315). The dominating tendency of laterals is exhibited also in pseudo-dichotomy. A lateral branch vein may often be as vigorous as its parent, which may, correspondingly, be deflected from its course, so that a symmetrical fork is produced, but not, however, a true dichotomy, since one arm of the fork is, in fact, the main vein, while the other is only a branch of it. (See Addendum.)

If the relation of an enation to its parent leaf is accepted as being of the same nature as the relation of the leaf itself to its parent shoot, we may regard the disproportionate corona of *Narcissus Bulbocodium* L. as an example in which a partial leaf dominates its parent whole leaf, just as a leaf may dominate its parent shoot.

From the preceding discussion the conclusion emerges that the parts of the leaf tend towards the status of the whole leaf; and similarly that the leaf tends to assume the status of the parent shoot; and, moreover, that this tendency is common to all the elements of the vegetative shoot complex. We have now to consider whether this attempt to rival the characters of the previous generation can be recognized in the leaf, in the crucial matter of reproduction. We may summarize the position by saying that while most leaves lack the capacity for giving rise to fresh shoots comparable with the axillary buds normally produced by shoots, yet such buds arise often enough to suggest that they are symptomatic of the urge to whole-shoot characters. In the tomato, *Solanum Lycopersicum* L., for instance, buds may occur in the "axils" of the large leaflets (Worsdell, 1915). It is probable that the reason why compound leaves do not more often develop "axillary" buds in connexion with their pinnae is because these pinnae, being developed from the junction line of the upper and lower surfaces of the leaf, lie in the plane of the leaf, and there is thus no angle between the rachis and the pinna corresponding to the angle between the stem and axillant leaf; and the lack of such an angle may perhaps be an impediment to budding. In leaves in general it is probable that the capacity for bud production is latent rather than actually absent. Leaf cuttings of many plants can be induced not only to root, but also to produce shoots, by appropriate cultural methods. Moreover, there are a number of examples of budding occurring as a normal event from detached leaves, and also from leaves *in situ* on the plant. For instance, in *Kalanchoë daigremontiana* R. Hamet et Perrier de la Bâthie, a native of Madagascar, adventitious plantlets are developed in the leaf notches, a situation which may have something in common with that in which axillary buds arise on shoots. The *Kalanchoë* buds are developed in the course of normal ontogeny, and can be detected when the leaves are from 2·5 to 3·0 mm. in length. Plantlet production is remarkably active; it is not uncommon for a large leaf to bear as many as sixty buds. While still attached to the parent leaf, the buds may themselves bear leaves which are already producing plantlets of the second order (Johnson, 1934). Another genus in which epiphyllous shoots are a frequent occurrence is *Drosera* (Vickery, 1933, etc.). The conditions of water life are specially favourable to vegetative growth, and hence to the development of buds from leaves. Among aquatics the Utricularias are particularly liable to bud development, and this may be associated with the fact that the leaves remain for a long time meristematic and capable of apical development (Goebel, 1889). Other well-known examples of aquatics which bud from their leaves are *Cardamine pratensis* L. and *Nymphaea stellata* Willd.

It is noticeable that, in all these cases of leaf budding, it is not a new leaf which is produced, but a new shoot, a fact which may be held to favour the view that the leaf is essentially of shoot nature.

An anomalous instance, the interpretation of which is still doubtful, but which serves at least to indicate the power of development latent in leaves, is that of the cotyledon of the unifoliate species of *Streptocarpus* (Hill, 1938). These species show no trace of a plumular bud between the cotyledons, one of which eventually dies. The survivor, by meristematic activity in its basal region, produces a single foliage leaf—the only one borne by the plant—which carries up the cotyledon at its apex; the leaf-meristem also gives rise to the flowering shoots and their bracts.

We have now seen that the leaf shows a tendency to advance towards being a whole-shoot, both in its competition with the parent shoot, and in its occasional reproductive activity. These may be regarded as points of behaviour. It remains to be seen whether a similar advance can be detected in the actual lay-out and structure of the leaf.

When we examine the dorsiventral petiole of a typical leaf, with its arc of bundles resembling in section a segment of the stem ring, we see that it has all the appearance of an incomplete shoot. Many petioles, however, are not simply dorsiventral, but show a pronounced tendency to become radial, or, in other words to advance structurally from the condition of a partial-shoot towards that of a whole-shoot. Anatomically this radialness may be achieved in a variety of ways. The simplest method is by the approximation of the margins of the arc to form a ring, e.g. *Helleborus* (Zimmermann, 1939); this ring may be completed by the fusion of branches from the marginal bundles to form one ventral bundle, e.g. *Clematis*

Vitalba L. (Gerresheim, 1913). Or, on the other hand, a second set of bundles may be given off from the bundles of the arc, as an upper series, inverted with respect to the main arc, and finally forming a ring in conjunction with the main arc (Zimmermann, 1939, p. 431, Fig. 10; throughout this diagram the *dorsal* bundle is wrongly labelled *ventral*). A related method of transition to radialness is that seen in *Albizzia julibrissin* Durazz., a member of the Leguminosae. In the base of the petiole, foliar traces (individually ring-like) form a three-membered arc. At a higher level this arc develops into a ring, the ventral bundles of which are formed from the marginal regions of each of the three traces (Watari, 1934). Yet another method of achieving radialness has been reported by Bouyges (1902), who finds that in *Aesculus Hippocastanum* L. and other plants, the ventral part of the vascular system of the petiole arises from a cortical meristem. There is still need of a comparative study synthesizing the various accounts of petiole structure, but it is at least clear that petioles are not characterized by one definite scheme of anatomy, but by an urge towards a certain goal—radialness—which may be reached indifferently by a variety of routes.

Among the monocotyledons many complete leaves show the same radial trend as the petioles of dicotyledons, a fact which led to the description of these leaves as "petiolar phyllodes" in earlier work (Arber, 1918, etc.; 1925, p. 100); but, in the light of more recent knowledge, it seems better to interpret them as *a more or less modified fixation of the whole phyllome at its pre-laminar stage.* In this phase the primordium often takes "the form of a tapering adaxially-flattened cone" (Foster, 1936), thus recalling a petiole, from which, however, it differs in so far as it is potentially a whole phyllome, whereas the petiole is merely one element in this ultimate whole.

In fully unifacial petioles the upper face is entirely eliminated, since the lateral junction lines of the upper and lower surfaces close around the petiole at the top of the leaf base, and meet in the upper median line. The whole external surface of the petiole thus corresponds to the external surface of the parent shoot, and if there is also a closed ring of bundles, the petiole may be held to have approached nearly to the radial character of the stem. The same approach is shown by the unifacial leaves of monocotyledons, which were long ago compared with stems by Trécul (1876).

It was pointed out by C. de Candolle (1899) that a certain type of lamina—the shield-shaped or peltate—tends to be associated with a petiole of unifacial form, which has "un système fibrovasculaire complet, c'est-à-dire également réparti tout autour de l'axe de figure". The relation between unifacial petioles and peltate leaves has been worked out in special detail by Troll, who gives 77 pages to the subject in his *Vergleichende Morphologie*. This will no doubt remain the standard factual treatment of the subject, but Troll's attitude seems to require some modification, since he regards the peltation of the lamina as *a consequence* ("eine Folge") of unifacial petiole structure (1932), whereas it seems more logical not to look upon the one as the cause of the other, but to think of both alike as expressions of the urge towards radialness, which in the petiole is translated into unifaciality, and, in the lamina, into peltation. That there is no invariable causal relation between these types of petiolar and laminar structure is indicated by the fact that there are a number of plants in which unifacial petioles are associated with non-peltate laminae. On the other hand, peltate laminae seem always to be associated with unifacial petioles, and, in the not infrequent examples in which a plant, which normally has non-peltate laminae and bifacial petioles, produces a peltate lamina as an abnormality, its petiole is found to have diverged from the form typical for the species, and to have become unifacial. Troll has shown (1939) that the double-bladedness of certain species of *Caltha* is a special case of peltation, and he has demonstrated by comparative study that the apparently bifacial petiole is really built on a unifacial plan.

A leaf with a peltate lamina, especially if it happens to be compound, often has a striking resemblance to a shoot axis bearing a distal tuft of leaves; Schultz-Schultzenstein long ago (1861) suggested this comparison for the compound-peltate leaf of *Lupinus*. This leaf, indeed, approximates closely to the construction of a shoot, in which, above a cluster of leaves, the growth of the apex has been inhibited. Other examples appear to point towards the same analogy. Those species of *Geranium* and *Pelargonium*, for instance, which have two stipules, and a petiole surrounded at the apex by a peltate lamina, may be compared with a flower (reproductive shoot) with two bracteoles, a pedicel, and a calyx surrounding the apex of the pedicel. Beyond the calyx no further comparison can be made, as the axis of the flower continues to grow and produces the remaining floral members, whereas lamina-production exhausts the resources of the leaf. Certain other leaves, which approach peltation without achieving it completely, are also strikingly shoot-like. The foliage leaf of *Eranthis hiemalis* L., for instance, with its long petiole and its spreading divided lamina, recalls the peduncle of the same plant, with its crown of three associated bracts; to complete the resemblance, one must suppose the flower to be undeveloped. In the peltate leaf of *Tropaeolum majus* L., shoot structure is imitated in a different respect—that of radialness—for here the leaf possesses no free bifacial region even in its base (Troll, 1933). As showing a hint of yet another shoot character, we may recall *Nymphaea stellata* Willd., in which, from the upper surface of the peltate lamina, directly above the attachment to the petiole, a shoot bud may develop, so that the whole structure may be compared, even if remotely, to a shoot with a growing apex.

Such examples as those just considered, seem to offer some justification for C. de Candolle's dictum that peltate leaves are "le type des phyllomes les plus développés". It is certain compound-pinnate leaves, however, which show the most obvious advance towards shoot structure. The rachis corresponds to the axis of the shoot, and the pinnae to the leaves, while the attachment levels of the pinnae recall shoot nodes, and may be distinguished as "nodelets". The anatomy of the rachis often shows a tendency towards the radialness typical of shoots. For instance in *Albizzia julibrissin* Durazz., which has a bipinnate leaf with 6–11 pairs of pinnae, consisting of numerous pinnules, not only is the rachis radial in structure, but the system in the base of each lateral pinna is also nearly radial (Watari, 1934). The arrangement and stance of the leaflets in pinnate leaves is, moreover, significant from our standpoint. Just as the distichous leaves of a shoot may be opposite (*Potamogeton densus* L.) or alternate (Gramineae), so the two rows of pinnae of a compound-pinnate leaf may be opposite or alternate. In bifacial pinnate leaves the placing of the pinnae is lateral (longitudinal), since they arise as outgrowths from the two edges of the rachis, i.e. from the intersection of the upper and lower surfaces. The pinnae are therefore all in the plane of the leaf; but as the rachis tends towards the axial character of unifaciality, the position of the pinnae changes. When the rachis is completely unifacial, i.e. when the leaf margins are in contact along the ventral median line, these margins separate only briefly at each nodelet, to allow of pinna formation, and then close again, the insertion of the pinnae thus becoming transversal (Troll, 1934). That is to say, as the rachis approaches the axis in its unifaciality, so the relation of the pinnae to the rachis comes increasingly to resemble that of leaves to an axis; and thus the leaf as a whole shows more and more approximation to a shoot. A highly organized form of this shoot-like scheme of rachis and pinnae is found in the umbellifer, *Carum verticillatum* Koch, in which the segments of the leaflets stand out from the rachis like a tufted whorl, the appearance of which must have suggested the specific name (Troll, 1939). The leaf as a whole recalls a shoot of *Ceratophyllum*, in which the whorling is again apparent only; but whereas it is due in *Ceratophyllum* to the sub-division of a pair of opposite *leaves* (Schaeppi, 1935), in *Carum* it comes about through subdivision of a pair of opposite *pinnae*. *Carum* shows another shoot-like character in the long continued growth in the apical region, which may result in a leaf over 40 cm. in length (Troll, 1934); as many as thirty-six "internodes" have been recorded (Glück, 1911). Prolonged meristematic activity near the apex is indeed found in various compound-pinnate leaves; a notable example is *Guarea* (Meliaceae), in which the youngest apical pinnae form a terminal "bud", thus giving the leaf a peculiarly shoot-like character (Troll, 1939).

A minor point of similarity between compound-pinnate leaves, and shoots, is that the pinnate leaf may, though rarely, have its rachis winged, e.g. *Filicium* (Sapindaceae) (Troll, 1939), thus recalling a shoot axis winged in one plane only, e.g. *Acacia alata* R.Br.

To the comparison of compound-pinnate leaves and shoots it may be objected that there is an essential difference in the order of development of the appendages, the leaves of a shoot being borne acropetally, while the pinnae of a pinnate leaf may be produced wholly or partially in basipetal order. The facts certainly seem at first glance to bear out this objection, but the possibility is not excluded that the type of leaf development which is called basipetal may be open to a somewhat different interpretation. In order to understand this point, we must give a wider consideration to the form of leaves. A general study of this subject brings out the striking fact that the leaf of the angiosperm has a very general tendency towards *ternation*, both external and anatomical. There are many examples of simple leaves with two main lateral veins in addition to the midrib, and of compound leaves with three leaflets, one median and two lateral. Moreover, various leaves which at maturity do not suggest ternation may be shown to be constructed on this plan. Foster (1929) describes the leaf of the horse-chestnut (*Aesculus Hippocastanum* L.) as passing through "a definite ternate condition", the remaining pairs of leaflets arising later through branching of the primary lateral pair. Compound-pinnate leaves do not seem at first glance to fall into a corresponding scheme, but Uittien (1928) has concluded that the apparently pinnate leaves of Umbelliferae are, in reality, ternate, the whole leaf, except the two lowest lateral branches, being, in his opinion, formed by the elaboration of the terminal pinna. It may, indeed, be suggested that there is at least a possibility of visualizing all compound-pinnate leaves as built on a ternate plan, though on lines somewhat different from those laid down by Uittien for the Umbelliferae. Compound-pinnate leaves fall into three categories—acropetal, basipetal, and a third group called divergent, in which the distal region is acropetal, and the proximal region basipetal (Troll, 1939). On the ternation theory, the pinnae of an acropetal leaf (or of the terminal acropetal region of a divergent leaf) may be interpreted as derived from a primary pair of lateral pinnae. These lateral pinnae give rise to a secondary pair of pinnae on the distal side, and these again to another pair on the distal side, and so on, as long as leaf development continues, each pair of pinnae being separated by intercalary growth from the pair from which it is derived. In a basipetal leaf (or the basipetal region of a divergent leaf) the reverse process happens. The primary lateral pair give off a pair on their proximal side, and these again give rise to another pair on their proximal side, and so on. Here again the nature of the process is masked by separation of the leaflets by intercalary growth. The difference between acropetal, basipetal, and divergent development depends upon whether the original pair of lateral pinnae, and their successive segments, branch upon the distal side, or the proximal side, or on both. If this theory be accepted, acropetal, basipetal, and divergent leaves will be recognized as modifications of the ternate leaf. The contrast, then, between compound-pinnate leaf development (ternation with serial derivative growth) and shoot development (acropetal growth) is a secondary result of the limitation of growth in the leaf apex. The leaf tip can do little in the way of forming appendages before its growth period ceases, and the capacity for further development suffers a compensatory transference to the outgrowths (pinnae or lobes), while, in the shoot, on the other hand, the apex continues to be actively meristematic, and thus forms successive outgrowths (leaves) in acropetal order.

V. THE BEARING OF THE PARTIAL-SHOOT THEORY ON THE HOMOLOGIES OF THE LAMINA

The prevalent type of ontogeny among dicotyledonous leaves is, as we have already noticed, the pleuroplastic, in which a rod-like rudiment gives rise to the flat lamina by growth from a marginal meristem. In some dicotyledons, however, the structure diverges widely from this type. In the so-called "midrib leaf" or "rachis leaf", for instance, found in *Plantago*, *Tragopogon*, *Scorzonera*, and various Umbelliferae, the rod-like pre-laminar leaf rudiment expands laterally, thus separating the veins (Gaisberg, 1922; Troll, 1939). There is nothing in such leaves, except perhaps a narrow edging, which is equivalent to the lamina wings of an ordinary pleuroplastic leaf. Among the monocotyledons, again, types of lamina still more unlike that of the pleuroplastic leaf, are the rule rather than the exception. In earlier work (Arber, 1918, 1922*b*) the view was taken that, when a blade occurred among the monocotyledons, it was not homologous with the dicotyledonous lamina, but was to be interpreted as a pseudo-lamina arising by transformation of the distal part of the petiole. This point of view needs some revision to-day in two respects. In the first place, one would prefer now to speak of the transformation of the pre-laminar leaf rudiment rather than of the petiole; and, secondly, though the *conception* of the pseudo-lamina retains a certain value as emphasizing differences from the pleuroplastic form, it seems best to give up the *name*, since it reflects the simple phylogenetic standpoint of its period and carries the implication that the pleuroplastic dicotyledonous lamina is the ancestral type, while that of the monocotyledon is derivative and imitative—an opinion which seemed natural twenty years ago, but which one would hesitate now to endorse.

The leaf of *Musa* may be taken as an example of one type of divergence from the pleuroplastic scheme. In this genus the lamina halves do not begin to develop until a comparatively late stage, when the marginal tissue of the rudiment has lost its meristematic character, and is thus incapable of serving as a source for wing development. So the meristematic layer forming the wing is supplied by deeper tissues, and the lamina halves developed from this meristem not only lie wholly within the scarious border, but even within the marginal bundles. Skutch, who in 1930 described the ontogeny of this leaf, concludes that the lamina halves are in no sense homologous with those of a dicotyledon.

As another very different example of a lamina which also contrasts sharply with the pleuroplastic type, we may take the strange foliated leaf of the irid, *Cypella*, which owes its elaborate construction to a process of invagination and winging, on a unifacial basis (Arber, 1921, Fig. 50H–N, pp. 321, 323). In the palms, again, invagination is one of the principal factors operative in producing the characteristic lamina (Arber, 1922*a*).

These examples show that any sound theory of the leaf must recognize the fact that certain elements of the foliage leaf in the angiosperms may have a diversified origin, and hence cannot always be neatly homologized from family to family. Now Troll and others, who adopt the type concept uncritically as the basis of their morphology, are accustomed to treat all leaves as referable ultimately to one type, so that they must all be amenable to analysis on a single unified scheme. This opinion follows logically if it is held that the leaf is a prime morphological category; but on the theory of the phyllome put forward in the present paper, this belief is discarded, and we are relieved of the necessity for forcing radically different structures into a framework of homology. For, according to our view, the need does not arise to visualize a process of leaf evolution traceable back to a primordial leaf. On the present interpretation there is no such thing as a primitive leaf type, from which all the existing forms of leaf have been derived, and thus there is no question of equating all leaves to such a primitive type and hence to one another. The leaf possesses no individual lineage *as a leaf*—from the first it has been neither more nor less than a special phase of the shoot system of the plant to which it belongs, so that its relation to the leaves of other groups is of an *indirect* kind. Its only *direct* relation is to the shoot of its own species.

VI. SHOOT AND ROOT

If the view is accepted that the leaf is a partial-shoot with an urge towards whole-shoot characters, we reach a unified picture of the shoot region of the vegetative body. It remains to be considered whether this picture can be extended to include the root. In a former paper (Arber, 1930) it was pointed out that shoot and root are alike in the fact that each gives rise to units like itself—the shoot to lateral shoots, and the root to lateral roots; the above-ground shoot complex, and the subterranean root complex are thus parallel developments. In the paper cited, root and shoot were treated as equivalent entities, but it is probable that this view to-day requires some modification. A peculiar feature of roots is that—unlike leaves—they take their origin deep in the tissues of the plant body; their radial symmetry may be connected with the protected and uniform conditions under which they arise. Their deep origin has an interesting sequel among periclinal chimaeras, in which root cuttings give rise to plants whose characters are found to be those of the inner component of the chimaera alone (Bateson, 1916, 1921). Now it has been suggested by Thoday (1939) in connexion with *Selaginella*, that "It is conceivable from a causal point of view that a rhizophore might be a sort of chimaera, with a core of root inside a skin of shoot". The hypocotyl of flowering plants, like a rhizophore, shows shoot and root characters distributed in a way which might be an indication of something like chimaera nature. May we, then, carry the idea further and hazard the highly tentative suggestion that the shoot itself may be compared to a periclinal chimaera, of which the inner component is of root nature? This idea would throw light on the fact that the root has no power of producing either leaves or sporogenous tissue, both of which normally arise from the more superficial layers of the shoot. In the earlier part of this essay we have been working out the conception of the leaf as a partial-shoot, imperfect in being merely a dorsiventral segment of the parent shoot; we are now suggesting that the root also is a partial-shoot, which is imperfect, not, like the leaf, in consequence of failure in apical growth, radial symmetry and self-reproductive power, but from *external* incompleteness. Like the leaf, the root shows a tendency to achieve whole-shoot structure; different as the young root may be from the shoot, its secondary thickening often produces a final result deceptively like the anatomy of an old shoot. If leaf and root are accepted as parallel in their relation to the shoot, it may be possible to draw a valid comparison between the anatomical transition from whole-shoot to leaf (partial-shoot) in the region of leaf attachment, and the transition from whole-shoot to root (partial-shoot) in the hypocotyl.

The supposition that the root may represent the "core" of the shoot is not intended to imply anything so naively simple as that the root is anatomically a continuation of the central region of the shoot alone; this, indeed, would be obviously untrue, at least for the main root. It is held, rather, that the root is endowed with tendencies and capacities which correspond to those of the inner region of the shoot rather than those of its surface. It is perhaps significant, that the root does, in fact, show a certain liability to divest itself of the layers corresponding to the external shoot layers. In monocotyledons, for instance, it is apparently only the root cap, and in dicotyledons, the piliferous layer also, which are direct continuations of the shoot epidermis, and these parts of the root are usually of ephemeral character (Tieghem, 1891; this work may, however, need checking with modern methods). Moreover, especially in dicotyledons, the original cortex is often at an early stage shelled off more or less completely by cork formation.

The speculative character of the conception of the root, which has just been sketched, is fully recognized, and the suggestion is thrown out merely as an indication of a direction from which the solution of the problem may eventually come.

VII. SUMMARY

The present essay is a sequel to an earlier study in this journal, dealing with the flower (Arber, 1937*a*). It is based upon the conception of the *shoot* as the basic unit, rather than the *stem* and *leaf*, or the *phyton* (cf. Arber, 1930). Troll's acceptance of root, stem, and leaf, as three unanalysable categories (1935, etc.) is criticized, and attention is drawn to a suggestion made by Kant in 1790, which appears to have been hitherto overlooked by botanists; this suggestion is that the leaf and lateral shoot are parallel members, each with a certain equivalence to the parent plant. It is maintained that Kant's idea has greater value to-day than Goethe's concept of the leaf as a primary category, which was promulgated in the same year. Casimir de Candolle's theory of 1868, which is in harmony with Kant's view, is reformulated as follows: the leaf is a partial-shoot, arising laterally from a parent whole-shoot. It is held, however, that this theory, though true as far as it goes, is incomplete. It is suggested that it can be developed to meet the facts by applying Spinoza's theorem of self-maintenance, which leads to the corollary that the leaf, as a partial-shoot, has a tendency to rise to the status of the parent shoot. The thesis advanced in the present paper is that: *the leaf is a partial-shoot, produced laterally from a parent whole-shoot, and revealing an inherent urge towards becoming a whole-shoot, but never actually attaining this goal, since radial symmetry and the powers of apical growth and of self-reproduction are curtailed or inhibited.* (It is explained that the use of such words as "endeavour" and "urge" must not be held to imply an anthropomorphic view of the plant.)

Most of the present essay is occupied with a study of the considerations which seem to make this theory an adequate way of visualizing the facts. The urge of the leaf towards whole-shoot characters is regarded, not as an isolated phenomenon, but as a part of a general tendency, recognizable throughout the shoot complex, which leads shoots of each generation to compete with and endeavour to replace the parent generation. In connexion with the relation of leaf and shoot, the suggestion is made that all compound-pinnate leaves, whether acropetal, basipetal, or divergent, may be regarded as variants of the ternate type. Ternation, and the serial pinna development which may follow it, are regarded as associated with the inhibition

of apical growth and the correlated occurrence of intercalary replacement-growth, which are among the features differentiating leaf from shoot.

The study of the leaf ends with a note upon the bearing of the partial-shoot theory upon the question of the homologies of laminae of different kinds. It is concluded that the morphological relation to one another of the leaves of different angiosperms is always indirect; their only direct relation is with the shoot of their own species, of which they are partial expressions.

The final problem, which is considered very briefly, is whether the root must be treated as a primary category, parallel and incommensurable with the shoot, or whether it is possible to relate it in any way to the shoot. Towards the solution of this problem, a suggestion is offered, but its tentative and speculative character is fully recognized. This suggestion is that *the shoot is in some degree analogous to a periclinal chimaera, with an internal component, which may be interpreted as of root nature*; on this hypothesis the root itself is comparable with the leaf, since they are both, in different ways, partial-shoots. The vegetative body, from this standpoint, consists entirely of shoots and partial-shoots, so that this theory offers an approach to a unified picture of plant construction in the angiosperms.

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For further notes on the apices of flowers and inflorescences (considered at the foot of p. 92) see Arber, A. (1940), "Studies in flower structure. VI. On the residual vascular tissue in the apices of reproductive shoots, with special reference to Lilaea and Amherstia." *Ann. Bot. Lond.*, N.S. **4**, 617–27.

A further example of the subordination of a main bundle to its two principal laterals, mentioned on p. 94, may be added from gynaeceum venation. It often happens that the ventral bundles of a carpel are much more important than the median strand. *Papaver* offers a striking instance, in which not only the bundles, but the whole carpel, shows this characteristic. The median region of the carpel is so much reduced that it is the margins alone which reach the apex and form the stigmas (Arber, 1938, "Studies in flower structure. IV". *Ann. Bot. Lond.*, N.S. **2**, 649–64).

THE FUNCTION AND METABOLISM OF CALCIUM IN THE INVERTEBRATA

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I. INTRODUCTION

CALCIUM is an essential constituent of protoplasm and of the body fluids of all animals. It is necessary for the normal activity of the neuromuscular system, for the maintenance of the normal permeability of cells and organisms, and for the stability of the matrices between cells. For normal coagulation of blood in vertebrates calcium ions are essential, and they also activate some enzymes, particularly pancreatic lipase. Perhaps on account of its abundance and its property of forming relatively insoluble compounds such as the carbonate and phosphate, calcium has been used in practically every phylum for the formation of internal and external skeletons.

Certain features of the metabolism and function of calcium in invertebrates are discussed in this review. It has not been possible to deal in detail with some aspects such as the role of calcium in muscular contraction and ionic antagonism, since adequate treatment would involve the whole subject of the action of electrolytes on tissues. A useful discussion of this is given by Heilbrunn (1937). For data on the chemical composition and mineralogical form of invertebrate skeletons the papers of Clarke & Wheeler (1922) and Prenant (1927[1]) may be consulted.

Animals find their sources of salts in their food, drinking water, or aquatic medium. The calcium of natural waters is derived ultimately from the solution by rain water of the calcium compounds in rocks and soils. The breakdown of plagioclase felspars, pyroxenes and hornblende, all complex calcium-containing silicates, and the solution of limestone rocks, chalk, apatite and gypsum all contribute to the calcium content of natural waters, and the abundance of these compounds in most soils is reflected by the usual predominance of calcium over the other cations in fresh water. The composition of some typical natural waters can be seen in Table I.

Table I. *Composition of some typical natural waters* (*g. per litre*)

	Na	K	Ca	Mg	Cl	SO_4	HCO_3
Sea water*	10·543	0·382	0·403	1·272	19·000	2·652	0·141
Hard fresh water (Wembury stream)†	0·021	0·016	0·064	0·014	0·041	0·024	0·242
Soft fresh water (Loch Katrine)‡	0·003	—	0·002	0·001	0·005	0·005	0·004

* Webb (1939). † Pantin (1931*a*). ‡ Thresh *et al.* (1933).

Why is calcium present in such relatively low concentration in sea water compared with sodium, potassium and magnesium? The reason lies in the insolubility of its carbonate in an alkaline medium. Vast quantities of calcium and probably carbonate ions have been withdrawn in past ages and are still being withdrawn by marine animals of practically every phylum and by coralline algae for skeleton formation. In addition, calcium carbonate is removed from sea water by direct physico-chemical precipitation and by precipitation resulting from the metabolism of bacteria (Clarke, 1924, pp. 131–2; Black, 1933). Calcareous sediments produced thus by both biological and physico-chemical agencies have formed chalk and limestone.

The calcium content of natural waters varies widely, from less than 1 mg. per litre in some acid peaty waters to about 120 mg. per litre in some chalky streams and rivers, and 400 mg. per litre in normal sea water. In some inland salt lakes with 5 times the salinity of sea water a calcium concentration 26 times that of sea water has been found (Pruthi, 1932).

II. THE PHYSIOLOGICAL ACTION OF CALCIUM

(1) *Effect on permeability of cells and organisms*

The importance of calcium in regulating the permeability of estuarine animals has been shown clearly. The platyhelminth *Procerodes* (*Gunda*) *ulvae* can live indefinitely in sea water, but its ability to live in fresh water depends on the concentration of salts and on the presence of calcium (Pantin, 1931*a*, *b*; Weil & Pantin, 1931; Beadle, 1934). If *Procerodes* is transferred to diluted sea water, it becomes swollen owing to an osmotic intake of water, loses some salts but survives.

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[1] *Biological Reviews.*

If placed in hard stream water containing 64 mg. Ca per litre it survives for a much longer time than when placed in Plymouth tap water which has only about 2 mg. Ca per litre. The longer survival in the hard water is associated with the less rapid loss of salts, and therefore the permeability of the surface membranes of *Procerodes* and its ability to survive in fresh water depend on the concentration of calcium in the external medium. In nature, *Procerodes* has been found in suitable streams down to a minimum calcium value of about 5 mg. per litre, and specimens are able to stand at least 5 days' exposure to water of 8–12 mg. per litre (Ritchie, 1934). The protective action of calcium added as calcium chloride has also been shown in another species of *Procerodes* (*P. wheatlandi*) when specimens are transferred to very hypotonic sea water (Oesting & Allee, 1935). In the case of a freshwater planarian, *Planaria dorotocephala*, a calcium concentration of 3 mg. per litre is sufficient to prevent the cytolytic disintegration which takes place in distilled water (Buchanan, 1935).

Nereis diversicolor, a polychaete worm, can survive immersion in brackish water. If specimens are transferred from pure sea water to a diluted one, they swell up owing to an osmotic intake of water, but survive (Schlieper, 1929) and expel some of the water by contracting their body-wall muscles (Beadle, 1937). The resistance of this species to immersion in brackish water depends on the presence of calcium ions (Ellis, 1933, 1937). If calcium is absent, the worms are unable to control their body volume in diluted sea water. Another consequence is the loss of hypertonicity of the body fluid (Beadle). This explains the uptake of water of worms kept in 20% sea water when transferred to calcium-free 20% sea water (Ellis, 1937). In sodium, potassium or magnesium-free sea water, this uptake does not take place. For the regulation of body volume all the cations of sea water are necessary (Ellis). This result can best be interpreted by assuming that these ions are essential for the muscular activity involved in the volume regulation.

A species of the isopod *Ligia* will survive hypotonic sea water (25%) much longer if extra calcium ions are added (Barnes, 1938). Permeability to all ions of the non-cellular chitinous lining of the foregut of *Homarus* is greatly reduced in the presence of calcium (Webb, 1940).

The property of calcium ions in reducing permeability to water is also known in cells. The unfertilized eggs of *Arbacia* absorb water if placed in a solution of glucose having the same osmotic pressure as 40% sea water, but if a little calcium or magnesium chloride is added to the non-electrolyte solution the intake of water is halved (McCutcheon & Lucké, 1928). If freshwater amoebae are transferred from a modified Ringer solution to distilled water, they increase in size. When placed in a hypertonic solution of lactose they decrease in volume. Both monovalent and divalent ions decrease this permeability to water, but calcium ions are the most effective (Mast & Fowler, 1938).

In general, the decrease in water and ionic permeability of cells and organisms in the presence of calcium appears to be a surface reaction, in which there is a threshold concentration above which little additional effect is produced. This control of permeability is of vital importance. The migration and colonization of fresh water by marine organisms, and the adaptation of estuarine organisms may depend to a large extent on the presence of calcium in sufficient concentration in the water (Weil & Pantin, 1931). Even marine fish can live in fresh water provided it is rich in calcium salts (Breder, 1934). Pruthi (1932) believes that the colonization by mayfly larvae (*Chloen* sp.) and hydrophilid beetles of salt waters of the Salt Range, Punjab, with salinities 2–5 times that of sea water is due to the control over permeability achieved by these insects in waters with 1·5–26 times the amount of calcium found in normal sea water. But experimental proof of this is still lacking.

(2) *Influence on stability of intercellular matrices and mucous coverings*

Of great importance is the fact that divalent ions such as calcium and magnesium stabilize intercellular matrices and the surfaces and mucous coverings of cells and organisms. Distilled water has a disintegrative effect on most freshwater animals, especially those with soft integuments. As long ago as 1894 Ringer & Sainsbury clearly established the protective action of minute amounts of calcium salts in preventing the disintegration of the freshwater oligochaete *Tubifex* in such a medium.

In calcium-free sea water, the hyaline layer does not form on the surface of an echinoderm egg (Gray, 1931, p. 107), while the matrix holding together the cells of a developing larva is dispersed (Herbst, 1900). The protoplasmic bridges between the blastomeres also break and cell division stops (Moore, 1932). Developing sponges do not fix themselves or undergo metamorphosis in calcium-free sea water, but eventually disintegrate by the separation of their cells. Under the same conditions the matrix between the cells of young metamorphosed sponges dissolves or loses its coherence, the cells wandering off (Maas, 1906). Reunion of the cells of the marine sponge *Microciona* which have been separated by squeezing the adult sponge through bolting silk will not take place in the absence of calcium or magnesium (Galtsoff, 1925). The coalescence of these cells results from amoeboid movement, and it is significant that marine limax amoebae are unable to move in a medium without calcium (Pantin, 1926). Apparently amoeboid movement of the sponge cells will take place if either of the divalent ions are present, but coalescence of the cells by their sticky outer hyaloplasm is complete only if both these ions are present.

In *Procerodes* dispersion of the mucus secreted by the animals occurs in calcium-free sea water, resulting in the loss of locomotion, since the planarians are unable to adhere to the substratum (Weil & Pantin, 1931). Mast (1929) has studied the attachment of *Amoeba proteus* to glass. Weak attachment can take place in distilled water, but firm attachment occurs if traces of the chlorides of sodium, potassium or calcium are added. In very dilute solution (e.g. *N*/40,000) calcium is much more efficient than sodium or potassium.

While a considerable part of these data indicates that most mucous coverings and intercellular matrices are probably calcium compounds, it seems from the work of Gray (1926) that the matrix between the cells on *Mytilus* gills is a mucoid protein stabilized particularly by magnesium ions. In the absence of magnesium, calcium ions precipitate the matrix in granular form.

(3) *Importance for neuromuscular system, amoeboid and ciliary movements, and other biological processes*

The tissues of an animal are adapted to live and function in an environment whose inorganic chemical composition resembles sea water, although the absolute concentrations of salts may be much less in some cases. The body walls of all marine invertebrates are permeable to sea water ions (references in Robertson, 1939), and it is not surprising that alteration of the external medium causes corresponding changes in the internal medium and various physiological effects on these organisms and their tissues. These effects have been studied by a few investigators, but a vast literature has grown up dealing especially with the results of altering the ionic balance of isolated tissues and organs. It is impossible to deal here with ionic antagonism, or the effects which alterations of calcium-ion concentration produce in practically all biological processes. One can only give some idea of the indispensability of calcium within certain concentrations for the normal reactions of animals and their tissues.

Calcium-free sea water, besides affecting the stability of intercellular matrices and mucous coverings, prevents the normal development of sponge and echinoderm larvae and formation of their skeletons (Maas, 1906; Herbst, 1904). But in all invertebrates studied—coelenterates, worms, crustaceans, tectibranch molluscs, ascidians, *Phoronis*, *Sagitta* and *Branchiostoma*—lack of calcium affects the neuromuscular system, causing a cessation of all rhythmical movements, loss of tone and flaccid paralysis of the muscles (Herbst, 1904; Bethe, 1927, 1928; Ellis, 1937). Crustacea such as *Carcinus* withstand such an artificial sea water for a much longer time than soft-bodied animals. This is because of the large calcium deposits in the tissues and exoskeleton which diffuse out slowly into the water. The normal calcium content of the blood is about 0·45–0·55 mg. per ml., but it is impossible to lower this to below about 0·10 mg. per ml. in calcium-free sea water. This low value causes greatly diminished muscular tone and serious impairment of the normal reflexes (Bethe, 1928). The effects of calcium-lack are reversible. If the calcium concentration is increased by adding calcium chloride to normal or artificial sea water, there is an acceleration of rhythmical movements and increase in excitability, which finally leads to spastic paralysis (Bethe, 1927, 1928). These effects of calcium on invertebrates are thus precisely the opposite of those found in vertebrates, where for example it is low blood calcium that is associated with hyper-excitability of nerves and muscular twitchings.

In marine organisms lack of calcium in the external medium causes the cessation of amoeboid movement and ciliary activity (Pantin, 1926; Gray, 1924), apparently because of its specific necessity for these processes. In the presence of monovalent cations, calcium is also necessary for locomotion in freshwater amoebae (Hopkins, 1929), but there are many complicated interacting factors concerned in this (Pitts & Mast, 1934). Chromatophore activity also is affected by the absence of calcium. In calcium-free sea water the shrimp *Crangon* is unable to concentrate its pigment granules when placed on a white background after being kept on a black one (Koller, 1930). Clotting of the coelomic fluid of *Arbacia* (Donnellon, 1938) and the blood of *Ligia* (Numanoi, 1939*c*) requires calcium, but it is unnecessary for the cellular clotting of insect blood (Mellanby, 1939[1]).

For normal activity of the invertebrate heart calcium within certain concentrations is necessary (Dubuisson, 1933). It is probably one of the essential elements for growth in invertebrates (see, e.g. Frost *et al.* 1936), and is present in most tissues (Webb, 1937). Despite what one would assume from the point of view of the ionic balance necessary for living tissues, Rubinstein *et al.* (1935) have reared *Drosophila* in a culture medium containing no sodium or calcium, and found that the experimental flies had only 5% of the sodium and 1% of the calcium present in controls reared normally. The mobility and viability of the flies were unaffected. In another series of experiments (Rubinstein *et al.* 1936) they were able to vary the Na : Ca molar ratio from 1·6 : 1 to 330 : 1. However, these results are exceptional.

III. THE FORMATION AND FUNCTION OF CALCAREOUS SKELETONS

(1) *Protozoa*

Calcareous skeletons are formed by many of the marine members of the Foraminifera. The underlying chitinoid membrane of the shell can be seen by passing carbon dioxide into the sea water in which they are living, thus dissolving the calcium carbonate (Doyle, 1935). This method should be useful for studying shell regeneration in these animals. Heron-Allen (1915) found that, on cultivating calcareous Foraminifera for several generations in small aquaria in which distilled water was added to counteract evaporation, the individuals tended to become transparent and "chitinous instead of calcareous" as the restricted amount of calcium became shared among larger numbers. The "chitinous" shell is of course the hyaline organic membrane. On the other hand, addition of hard water to counteract evaporation resulted in the formation of marked varieties characterized by the development of striae, ridges and teeth on the shells.

To a considerable extent, therefore, the degree of calcification of Foraminifera shells is dependent on the calcium content of the external medium.

(2) *Porifera*

The calcareous sponges are strengthened by a network of spicules, each of which consists of calcium carbonate in the form of calcite and an axial fibre and surrounding sheath of organic matter. Although these spicules arise intracellularly, they frequently become larger than the cells which secrete them. If this happens, the secreting cells may be found apposed to the sides of the spicule or at its ends, and the secretion of calcareous material has now become extracellular (Minchin, 1909).

Some experimental work has been done on the spicules of sponge larvae. Maas (1904) found that carbonate ions were necessary for the formation of the spicules, since larvae reared in sea water made carbonate-free by evaporation and re-solution did not produce spicules. In a synthetic carbonate-free sea water the same result was obtained (Maas, 1906). If normally metamorphosed young sponges were placed in such a solution, their spicules regressed even although calcium was present. These experiments indicate that for spicule formation carbonate ions other than those which can be derived from metabolic carbon dioxide are essential.

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[1] *Biological Reviews.*

The facts that regression of spicules only took place in living sponges and not in dead ones, and that isolated spicules did not dissolve in either of the sea waters argue against the only serious criticism of Maas's experiments, that the results may have been at least partly due to increased hydrogen-ion concentration of his experimental solutions compared with normal sea water (cf. Bouxin, 1926). The regression of the spicules in living sponges was probably due to increased acidity brought about by the respiratory carbon dioxide of the cells.

(3) *Coelenterata*

A large part is played by members of the Hydrocorallina, Alcyonaria and Madreporaria in the formation of the coral reefs which are such a feature of tropical seas.

Alcyonarian skeletons with the exception of that of *Heliopora* are built up of spicules of calcite which are secreted in the interior of certain ectoderm cells which have usually migrated into the mesogloea layer (Bourne, 1899). *Heliopora*, the hydrocorallines and the Madreporaria all form skeletons of aragonite (Prenant, 1927), secreted by the ectoderm, but the actual mechanism of the secretion has been disputed. Some early investigators believed that skeletons were formed by chemical precipitation between the ammonium carbonate secreted by organisms and the calcium sulphate of sea water. Murray & Irvine (1889), for example, maintained that the calcium carbonate in sea water was used by corals, and that the chemical reaction between ammonium carbonate arising from the decomposition of animal products and the calcium sulphate of sea water would result in making available all the calcium of the sea as the carbonate salt. They believed that the skeletons were secreted by the protoplasm, and that the chemical reaction referred to might take place within the tissues. Although the ionic theory has rendered valueless all discussions as to the availability of such arbitrary combinations of ions as calcium sulphate and calcium carbonate, the essential idea that the carbonate radicle of the skeleton may often be derived from metabolic carbon dioxide remains.

Cytologically, calcium has been shown to be present in certain cells of the ectoderm called calcoblasts (von Heider, 1881, 1886; Ogilvie, 1896; Hayashi, 1937, etc.), but disagreement centres around the relationship of these cells to the actual skeleton. According to Ogilvie (1896) the laminar structure of the madreporarian skeleton is due to successive layers of calcified cells (calcoblasts) which are continuously shed from the ectodermal layers. But Bourne (1899), using polarized light, searched without success for any sign of crystals or spicules of calcium carbonate within cells, and supported von Koch's (1882) finding that the madreporarian skeleton is deposited outside the living tissues. Matthai (1918) was diffident about accepting this extraprotoplasmic origin of the skeleton, on the grounds that it would be difficult to account for the many patterns of coralla formed without the more intimate intraprotoplasmic origin.

As far as present evidence goes, it seems that the madreporarian skeleton is formed by the syncytial ectoderm which secretes an organic matrix containing calcium carbonate in solution which crystallizes out soon afterwards (Bourne, 1899; Duerden, 1904; Hayashi, 1937).

(4) *Annelida*

Among the polychaetes, the members of the family Serpulidae secrete tubes of calcium carbonate. If worms are removed from their tubes, they commence to reform them, but rarely succeed completely (Harms, 1912; Faouzi, 1931). F. A. Potts found that the apparently homogeneous tube of *Pomatoceros triqueter* was composed of successive rings of calcium carbonate, and consisted of a mucoid base containing aragonite crystals (Robertson & Pantin, 1938). Partial reformation of the tube took place in artificial sea water of both normal composition and containing half the normal quantity of calcium (i.e. about 210 mg. Ca per litre), but failed in calcium-free sea water. While these experiments tend to demonstrate that the calcium for the formation of the calcareous rings is absorbed from sea water, it was still possible that the source may have been the calcareous plate in the operculum (Benham, 1910), and that in calcium-free sea water any mucoid and calcareous material secreted by the peristomial collar would be dispersed. I therefore analysed batches of worms before and after calcareous secretion had taken place in filtered natural sea water. The results (unpublished) demonstrate uptake of calcium from the water.

(5) *Arthropoda*

The possession of a stiff outer covering of chitin secreted by the epidermis is one of the features of the Arthropoda. In many of the higher Crustacea this covering is hardened by the deposition in it of calcium salts, chiefly the carbonate and phosphate, forming a protective exoskeleton. The relative rigidity of this skeleton makes increase in size impossible except at the moult, when the new skin which has been formed under the old integument is still soft and extensible. While the whole process of ecdysis or moulting is of great interest physiologically, the withdrawal of material before the old skeleton is cast and the rapid calcification of the new chitin are outstanding features. In arthropods generally, there is a large amount of resorption of organic matter prior to ecdysis (Wigglesworth, 1933; Drach, 1935*a*, 1935*b*, and others), while in those Crustacea with calcified skeletons, calcium salts may also be withdrawn (see e.g. Numanoi, 1934*b*; Robertson, 1937).

Ostracoda. The larvae of freshwater ostracods are able to harden their shells in calcium-free water. A special calcium carbonate reserve is laid down previously by the epidermis, after formation of the normal calcified shell; when the old shell has been moulted, this reserve undergoes solution, being used to calcify and strengthen the new shell (Fassbinder, 1912).

Amphipoda. Freshwater amphipods such as *Gammarus pulex* can live only in waters of suitable *p*H and salt concentration. At least 5 mg. Ca per litre is essential for this species. Schumann (1928) showed that certain gammarids including *G. pulex* were unable to harden their integuments and died in natural waters or bicarbonate solutions containing less than this amount of calcium. On the other hand, in calcium-rich waters the integuments of newly moulted animals were hard within 72 hr., being calcified in that time to practically the same extent as normal animals. Immediately after moulting, calcium is withdrawn from the water by the gammarids to be used for the calcification of their soft new skins. In calcium-poor water the addition of plants such as *Lemna*, *Cladophora*, and *Chara* enabled partial calcification to be attained in a few cases.

Isopoda. In marine isopods such as *Ligia*, the integument is cast in two portions (Tait, 1917; Numanoi, 1934*a*). Just before the posterior half of the carapace is cast, a "chalky whiteness" due to calcium carbonate (Herold, 1913; Nicholls, 1931) appears in the ventral skin of the anterior thoracic segments. This represents material which has been resorbed from the hind portion of the integument which is soon cast. Then the pleopods of the abdominal segments develop this chalky whiteness and the anterior portion of the integument is cast. The whiteness then disappears as the front portion of the new integument is hardened. Owing to this resorption *Ligia exotica* loses with its cast skin only about 25% of the original amount of calcium present in it (Numanoi, 1934*b*).

Decapoda. In *Ligia* no storage of calcium in the hepatopancreas or other tissues prior to moulting was found. But in the suborder Brachyura of the decapod Crustacea large amounts of calcium, up to 10–12% of the dry weight, may be stored in the hepatopancreas prior to moulting, and these are used in the formation of the new skeleton (v. Schönborn, 1912; Paul & Sharpe, 1916; Robertson 1937; Drach, 1939). Most of the calcium, in *Carcinus* at least, is present in the hepatopancreas as spherules of calcium phosphate. There can be no doubt that the calcium salts of the hepatopancreas and to a much less extent the other soft tissues are used in the hardening of the new skeleton, since calcification proceeds simultaneously with the depletion of the calcium reserves. In a *Carcinus* of 88 g. analysed a week after moulting, over 40% of the calcium in the hardening skeleton had come from the soft tissues of the crab, the remainder having been absorbed from the surrounding sea water (Robertson, 1937).

Soft post-moult crabs are able to absorb calcium ions from both normal and 50% sea water. In the former case, it appears that the process of diffusion could account for most of the uptake, since the blood calcium concentration of these *Carcinus* was lower than the calcium concentration of the surrounding sea water, but in 50% sea water or further dilutions, it would seem that uptake of calcium ions is an active process involving the expenditure of energy (cf. uptake of chloride ions in freshwater animals—Krogh, 1938).

Curiously enough, the members of the suborders Anomura and Astacura do not appear to store any calcium salts in the hepatopancreas at all. But in the Astacura (lobsters and crayfish) the peculiar structures known as gastroliths are present. These are plano-convex masses of calcium carbonate which are found in the foregut, one on each side, immediately prior to the moult. The gastroliths represent material which has been resorbed from the skeleton, and in *Astacus* they measure about 8 mm. just before ecdysis. A few hours after moulting they decrease in size and finally disappear (Damboviceanu, 1932), being apparently used as a source of calcium for the new skeleton. *Astacus* also withdraws calcium directly from the surrounding water to harden its integument (Schäperclaus, 1925).

In lobsters the gastroliths can form only a small reserve for the new integument. Calcium is probably absorbed from sea water in considerable amount immediately after moulting, and when the appendages are sufficiently hardened the cast skeleton itself may sometimes be eaten. It is eaten under aquarium conditions (Elmhirst, 1930).

Gastrolith formation occurs also in a species of *Sesarma* (Brachyura). When this calcium reserve is being dissolved after moulting prior to be reprecipitated in the integument, the blood calcium is raised to over 40 times its normal level (Numanoi, 1939*b*).

In some of Koller's (1930) experiments, there is some evidence for a connexion between the colour-change hormones and moulting in decapod Crustacea. When the "white glands" in the eye-stalks of the shrimp *Crangon vulgaris* were cauterized, the moulted skins weighed less than skins from normal shrimps of the same size, whether the animals were kept in diffuse light or in the dark. The reduced weight was shown to be due to less calcium carbonate, but it could not be decided whether this was the result of more resorption or less deposition of this substance than in normal animals. Hanström's (1939) observation that extirpation of the eyes in decapods results in earlier moulting than in normal animals suggests that the latter alternative is the more probable—the deposition of calcium carbonate in the integument will be less in animals which moult prematurely. The whole subject of possible hormonal control of calcium metabolism in invertebrates awaits investigation.

Insecta. In the Insecta, calcium has been used as a strengthening agent in the Orthoptera. Embedded in the horny egg-case of the cockroach are numerous crystals of calcium oxalate produced by the left colleterial gland (Ito, 1924), but it is doubtful whether these crystals have anything to do with the hardening of the case (Pryor, 1940). The egg-shells of stick insects contain calcium carbonate derived from stores previously accumulated in the Malpighian tubes of the female (Pantel 1919). The calcium (as Ca) amounts to about 23 % of the total dry weight of the egg-shell (Robertson, unpublished), a value comparable to one of about 37% in the hen's egg (Needham, 1931, p. 1264). In birds and reptiles, a transference of calcium from the egg-shell to the embryo occurs during development (Needham), but whether this takes place in the stick insect egg is unknown.

Only in a few Diptera is the exoskeleton calcareous. In stratiomyid larvae, such as *Sargus*, calcium carbonate is deposited in the integument after each moult. The deposit takes the form of groups of calcareous warts embedded in flat pits, and according to Müller (1925) it is derived from the calcium carbonate present in the Malpighian tubes. On analogy with the crustacean skeleton, this author postulates resorption of calcium from the old skin before it is moulted, but definite evidence is lacking. In some psychodid larvae such as *Pericoma* only the spines are calcified. The larvae are found in calcium-rich springs (168–255 mg. Ca per litre) which according to Krüper (1930) are the direct source of the calcareous material.

In many phytophagous and saprophagous dipterous larvae, calcium carbonate occurs in the Malpighian tubes or the fat body in the form of small granules or large calcospherites. During pupation these deposits are dissolved in the perivisceral fluid and pass in solution to the ecdysial fluid outside the new pupal cuticle. By withdrawal of the ecdysial fluid, the calcium carbonate is deposited on the inner surface of the old larval cuticle now forming the puparium (Keilin, 1921). The larvae of many cerambycid beetles which burrow in the wood of trees accumulate calcium carbonate in their Malpighian tubes towards the end of their feeding period. This is used together with a silky, gummy secretion to form opercula closing the pupal cell. Some species line the walls of the cells also with this calcareous secretion (Mayet, 1896; Beeson, 1919). By this means protection is afforded against natural enemies such as insect predators and parasitic fungi, while a suitable moisture content is maintained inside the chamber.

(6) *Mollusca*

Formation of shell. The shells of molluscs consist of an outer organic membrane of conchiolin, the periostracum, and a series of crystalline layers of calcium carbonate which also contain conchiolin. The innermost layer is formed by the epithelium of the entire mantle and the visceral mass, the outer layers and the periostracum usually by the epithelium of the mantle edge alone. Under certain conditions the periostracum may be formed by the whole mantle epithelium, for example in the regeneration which occurs after total removal of the shell (Boutan, 1923).

Many points about the secretion of the shell are still obscure (Kessel, 1933). There is general agreement that calcium carbonate is separated from the blood by certain cells of the mantle edge as spherules or granules which are liberated in colloidal form between the epithelium and the periostracum. Crystallization from this colloidal gel takes place therefore outside the cells themselves (Crofts, 1929; Dakin, 1912; Kessel, 1933). The gland cells which secrete calcium carbonate may be subepithelial and not epithelial, in some cases at least (Davis & Fleure, 1903). Prenant (1924, 1928) has shown that in the formation of the epiphragm in *Helix pomatia* the cells concerned are leucocytes located in the connective tissue of the mantle edge. The carbonate secretion appears first in these cells in amorphous form, soon changing secondarily into vaterite spherules. The contents of the cells are then discharged across the mantle epithelium. In the fully formed epiphragm the unstable vaterite changes to calcite.

Pearls have the same chemical composition as the innermost shell layer (nacre). They are formed in little sacs on the external face of the mantle. The calcareous material arises in special amoeboid cells which traverse the mantle epithelium. The latter forms the concentric layers of conchiolin (Boutan, 1923).

The conception of shell formation given above has been challenged by de Waele (1930) whose work has been accepted by recent authors (Manigault, 1933; Dotterweich & Elssner, 1935). De Waele has shown that an extrapalleal fluid is present in *Anodonta* between the mantle and the shell. It amounts to about 25 % of the weight of the tissues and is chemically identical with the blood. If exposed to the air, a precipitate consisting of albumen and calcospherites is formed. From this it is deduced that shell formation is a purely physico-chemical process, and that the calcium of the blood and the extrapalleal fluid is a part of a complex compound containing protein, carbon dioxide, and calcium carbonate which splits into its components by escape of the carbon dioxide.

Precipitation of calcium carbonate from this colloidal solution will depend on the tension of carbon dioxide in the immediate atmosphere. Dotterweich & Elssner (1935) found that calcium carbonate crystals were formed in the extrapalleal fluid only in an atmosphere containing less than 1·5% carbon dioxide. But Manigault (1933), also working on *Anodonta cygnea*, has shown that shell regeneration will take place in atmospheres containing carbon dioxide up to 15%. Dissociation of the hypothetical protein complex should have been quite impossible on physico-chemical grounds. This proves clearly the inadequacy of de Waele's conception. The cells of the mantle must be taken into consideration. When active shell secretion is taking place, calcium carbonate and associated organic matter will be liberated between the mantle and shell from cells in which they have accumulated (see e.g. Crofts, 1929). Probably the crystallization of calcium carbonate from this colloidal solution and its precise mineralogical form depend on purely physico-chemical conditions, but these have not yet been defined. De Waele's complex protein containing carbon dioxide and calcium carbonate is still without real chemical basis. It is unlikely that the whole of the calcium in the fluids under consideration is a calcium proteinate of this kind. Further, the calcospherites and precipitated albumen formed in his experiments from blood and extrapalleal fluid contained 58% organic matter, whereas the shell contains only 4%. This would necessitate such a large resorption of organic matter from new shell formed in this manner that the whole process as visualized by him is highly improbable.

Origin of shell material. The greater part of the calcareous material of the shells of marine molluscs is probably absorbed directly from sea water as calcium and bicarbonate ions which are secreted as calcium carbonate by the mantle edge. Metabolic carbon dioxide, however, will be the primary source of the carbonate radicle.

Orton (1925) found that shell growth in oysters could take place in sterile sea water in the practical absence of food. In a study of oysters by Galtsoff (1934) in Long Island Sound, the soft tissue weight increased from 6 to 14 g. with a corresponding increase in the shell weight of 60–100 g. from the month of May to November. From November to the next May the soft tissue weight remained practically stationary, being 15 g. towards the end of April, but the shell weight had increased to about 135 g. at practically the same rate as before. During most of this period, the temperature of the water was below 7° C. and the oysters were in a state of hibernation, no trace of food being found in the digestive tract.

From an analysis of oyster shell (Chatin & Muntz, 1895), it can be calculated that the 75 g. of shell formed in 12 months corresponds to about 27 g. calcium, and to an absorption of 520 mg. calcium per week. Compared with an absorption of 187 mg.

per week by a soft post-moult *Carcinus* weighing 88 g. (Robertson, 1937), this is an exceedingly large amount, and a study of the mechanism of the absorption is very desirable. Preliminary experiments made by me during March and April 1940, at a temperature of 10° C., have failed to show direct uptake of calcium ions from sea water in certain common gasteropods and bivalves. This result may only indicate that the proper environmental and physiological conditions were not attained by the molluscs in the experiments.

Under English estuarine conditions, Orton (1928) has found in *Ostrea edulis* that visible increases in shell area occur in spring and autumn when food is abundant and the temperature of the water lies between 10 and 16° C. In view of Galtsoff's (1934) results, it cannot be conclusively accepted that shell growth here does not occur below a temperature of 10° C., especially since possible increase in shell thickness was not investigated. In some bivalves such as *Cardium edule* concentric rings or grooves are present on the shell. Some of these represent the reduced winter growth of shell area, others are disturbance rings (Orton, 1926).

As far as freshwater molluscs are concerned, conclusive proof is not yet available that the calcium of the water may be directly utilized in shell formation. Thin-shelled specimens of the mollusc *Limnaea pereger*, if bred in hard water, respond by thickening the shell (Boycott, 1936). Adding calcium salts such as the carbonate, sulphate, and phosphate to pond water causes greater shell growth in specimens of *L. columella* than in controls kept without these salts (Colton, 1908). But in experiments of this type, it is often uncertain whether the greater shell growth is due to the higher calcium content of the food plants or to direct uptake of calcium from the water, or to a combination of the two. That *Anodonta* and some pulmonate molluscs do at least absorb calcium from fresh water has been shown by de Vorges (1903).

The difficulties of separating and controlling the factors concerned in shell growth have been responsible for the many interpretations of the old experiments of Semper (1881). Semper allowed equal numbers of *Limnaea stagnalis* eggs from the same batch (i.e. from the same parent) to develop in unequal volumes of water. The larger the volume of water up to a certain limit (about 4·5 litres), the larger were the shells of the snails; in small volumes of water the snails remained dwarfs. Since he believed that all other conditions such as temperature, light, and especially the food supply were constant, he thought that some chemical substance connected with growth must be absorbed from the water, and in the small volumes of water it would soon become depleted. This substance might have been calcium, although Semper himself did not know what it was. These experiments have been repeated by several investigators. De Varigny (1894) concluded that the dwarfing of the snails in small volumes of water was due to lack of exercise and movement, Willem (1896) that it was due to lack of aeration, and Legendre (1908) that it was caused by an accumulation of excretory products. Colton's (1908) experiments confirmed the importance of aeration, which probably acts by oxidizing harmful waste products of metabolism, which are of course more concentrated in small volumes of water. Food and the presence of calcium salts were found to be important factors in increasing shell growth. Popovici-Baznosanu (1921) and Turner (1927) conclude that lack of food was probably the chief factor responsible for Semper's results. Small volumes of water have no dwarfing effect if sufficient algal microflora is supplied. Insufficiency of food and aeration are the commonest factors inhibiting shell growth (Crabb, 1929).

As a general rule molluscan species which live in hard water have larger and heavier shells than those living in soft water, while the same holds true of different specimens of the same species (Boycott, 1936; Carpenter, 1928; Morrison, 1932). When it is considered that heavy well-calcified shells are characteristic of marine species living in a medium containing about 400 mg. calcium per litre, it is clear that optimum environmental conditions for shell growth are not present in many freshwater habitats.

The effect of temperature on the larval shell growth of *Limnaea* has been studied by Imai (1937). Although there is more rapid growth and development of the mollusc at higher temperatures, 23 and 28° C., the final shell size is greatest at a lower temperature, 18° C. From a study by Baldwin (1935) of the respiratory quotient of developing *Limnaea* eggs, it is very probable that the carbonate for shell formation is obtained by retention of metabolic carbon dioxide.

Whether certain stores of calcium present in the soft tissues of some freshwater bivalves have any importance with regard to the shell is unknown. In the different soft parts (mantle, gill, visceral sac) of *Cristaria*, Numanoi (1939*a*) has found calcium to be more than 10 times as concentrated as in the corresponding parts of marine bivalves. Over 9% of the dry weight of the gills consists of calcium in a form not yet determined.

The egg capsules of certain aquatic Neritidae (Streptoneura) are hardened by calcareous spherulites stored previously in a special sac opening into the oviduct. According to Andrews (1935, 1937) these have come from the digestive gland, and in the genus *Nerita* they are sufficiently distinctive to be of use as specific characters.

Field naturalists have long been aware that many species of terrestrial snails prefer a habitat with an abundant supply of calcium carbonate. From experiments by Oldham (1929, 1934), it is clear that the thickness and weight of snail shells is dependent on the snail's opportunity of obtaining calcium salts in suitable form. Secretion of shell material goes on almost automatically provided sufficient calcium compounds are available (cf. calcium secretion in earthworms, § IV, p. 124). In one of Oldham's (1934) experiments, young *Helix aspersa* of the same size were divided into two groups, one of which was fed on cabbage, oatmeal and chalk, and the other on cabbage and oatmeal alone. One year later, those fed on the chalk diet had shells 4·5 times as heavy and a little larger than those without chalk. The shell weight of such molluscs as *H. pomatia* under natural conditions was also found to be directly correlated with the calcium content of their environment.

In most of the pulmonate molluscs, the land snails and slugs, the eggs which are laid in the soil are enclosed in a hard calcareous shell. Carrick (1938, and personal communication), working on the embryology of *Agriolimax agrestis*, a common slug, has observed that the shell becomes progressively thinner and more transparent as the embryo inside develops and itself acquires a shell. This is worth following chemically, since it parallels the transfer of calcium from the egg-shells of terrestrial reptiles and birds to the developing embryo (Needham, 1931, pp. 1260–71). In reptiles and birds, the calcium is withdrawn from the shell as the bicarbonate by means of the allantoic blood vessels, and is used chiefly for the developing skeleton of the embryo.

Shell repair in *Helix pomatia* has been investigated from the experimental and chemical points of view by Sioli (1935). If a piece of shell about 2–3 sq. cm. in area is removed from the mollusc without damage to the underlying epithelium, a new piece of shell is regenerated. To determine the stimulus which initiated shell repair, the exposed epithelium was excluded from the light, prevented from drying, and the weight of the missing part imitated by pieces of glass and gelatin. Since shell repair went on under all these conditions, Sioli concludes that the stimulus must have been the break in the chemical balance between the epithelium and the shell.

By analysis of the tissues of normal snails and those which had been allowed to regenerate their shells for 4 weeks, he tried to determine the source of the calcium required for the repair. The ash content of both the liver and the kidney fell during the repair, presumably owing to the utilization of calcium. A peculiar point was that the phosphorus content of the liver did not decrease; it appears that the calcium phosphate spherules, the form in which most of the calcium and phosphorus exists in the liver, must break down, only the calcium being utilized and the phosphate remaining in the liver, perhaps in organic combination. There was no decrease in the ash content of the mantle and the visceral sac epithelia, and the calcium content of the foot did not alter significantly. He could now calculate the amounts of calcium in the liver and kidney which had been mobilized for shell repair and compare that amount with the calcium present in the regenerated pieces of shell. Of a piece of regenerated shell containing 344 mg. calcium, only 56 and 15 mg. were available from the liver and kidney respectively. Thus only about 20% could have come from these organs, and the rest must have come from other parts of the shell. A direct attempt to measure the amount of calcium which must come from the shell by removing a piece of the shell, weighing it, sticking it back on and reweighing it after 12 days was unsuccessful, as the piece actually increased in weight. The epithelium had regenerated a new piece round the edge.

The mobilization of the calcium stores in the liver was not due to an increased acidity, since the *p*H of liver extract and of frozen sections did not change in the animals which were repairing their shells. The *p*H of about 5 which is present in the cells of the digestive gland would be expected to dissolve tertiary calcium phosphate, but the crystals are apparently protected by an organic membrane. During regeneration, an enzyme may dissolve off the organic membrane round the crystals, liberating the calcium in ionic form or as calcium proteinate, the phosphate remaining in the gland in organic combination.

Function of shell. The shells of molluscs have not to be regarded as rigid protective coverings only. Besides acting as barriers to free diffusion of ions between the soft tissues and the environment, they play an important part in the regulation of the acid-base balance of the blood.

If tidal zone molluscs are placed in an atmosphere of air, there is an increase in the bicarbonate and calcium content of the blood with no alteration or but a very slight decrease in *p*H (Collip, 1920, 1921). Kokubo (1929), for example, found in the oyster that 2 day's air exposure caused a decrease in blood *p*H from 7·45 to 7·00, while the carbon dioxide content of the blood in volumes per cent. increased from 4·35 to 11·46. After a week, the *p*H of one specimen was 6·4, and the carbon dioxide 40·19 vol. %. Under these conditions the molluscs are respiring anaerobically (Collip; Kokubo; Kühnelt, 1938), and it was assumed that the metabolic carbon dioxide produced reacted with the calcium carbonate of the shell to produce the bicarbonate which buffered the blood:

$$CaCO_3 + CO_2 + H_2O \rightarrow Ca(HCO_3)_2.$$

Erosion of the shell is clearly visible (Kühnelt; Dugal, 1939), and is brought about not by the mantle edge but by the more central part of the mantle which increases in combined carbon dioxide content (Dugal). Dugal has concluded that the bicarbonate is produced not by reaction with carbonic acid, but by reaction between the calcium carbonate and an organic acid, probably lactic acid, produced during anaerobic glycolysis:

$$2CaCO_3 + 2Hl \rightarrow Ca(HCO_3)_2 + Cal_2.$$

He has shown that in *Venus mercenaria* a non-volatile weak acid increases in concentration in the mantle cavity fluid along with the combined carbon dioxide and calcium, and that the sum of the carbon dioxide and this acid is at any time equivalent to the calcium.

But when *Anodonta* is kept under anaerobic conditions the bicarbonate concentration increases more rapidly than the calcium concentration, rising from 60% of the latter to about 115% in 2 days. Since the *p*H remains constant in anaerobiosis, the excess of carbon dioxide not bound with the shell as calcium bicarbonate must be bound by some other means, probably by the calcium albuminate of the blood, which therefore is a further component in the buffering system (Dotterweich & Elssner, 1935).

Part of the difference between Dugal's and Dotterweich and Elssner's results may be due to the conditions of the experiments. Dugal investigated the mantle cavity fluid of molluscs kept out of water, whereas the other two authors were investigating the blood of molluscs kept in a vessel in water under paraffin oil. Diffusion of bicarbonate and escape of carbon dioxide were thus occurring under completely different conditions. Whether both carbonic acid and an organic acid such as lactic acid are produced under anaerobic conditions, or only one of these can be decided only by future work. The essential point is that such acids are buffered by the calcium carbonate of the shell, and do not produce significant increases in the hydrogen-ion concentration of the blood.

(7) *Echinodermata*

The spicular skeleton of larval echinoderms is formed by the mesenchyme cells which arise just after gastrulation. Direct dependence on sea water for the salts or ions used for the spicules is shown by the fact that the latter will not develop in sea water in which the calcium has been seriously reduced (Pouchet & Chabry, 1889) or in which calcium and carbonate ions are absent. The presence of magnesium and sulphate ions is also necessary for spicule formation (Herbst, 1904).

According to Rapkine & Prenant (1925), the *p*H of the blastocoele fluid of *Paracentrotus lividus* rises abruptly when the spicules first appear, reaching a *p*H of 8·5, and then it gradually falls to its initial value, that of the surrounding sea water. They consider that the rise is due to the absorption of carbon dioxide by the scleroblasts, the mesenchyme cells associated with the developing skeleton. This carbon dioxide is then joined with calcium to form the calcium carbonate of the spicules. It has been shown also (Vlès & Gex, 1925) that if developing eggs are isolated in a small volume of sea water to which cresol red has been added as indicator, the *p*H of the water at first falls owing to the excretion of respiratory carbon dioxide by the larvae, but, coinciding with the formation of the spicules, the *p*H then rises markedly, apparently because of actual absorption of carbon dioxide by the larvae. Thus, when the spicules are being formed, metabolic carbon dioxide alone is insufficient to furnish the carbonate radicle, and bicarbonate ions from the surrounding sea water are absorbed in addition to calcium ions.

Although Chambers & Pollack (1927) did not confirm the rise in *p*H at gastrulation, Hirabayashi (1937) has confirmed this, finding a *p*H at gastrulation of 8·4–8·6, compared with 8·3 for the surrounding medium. The larvae of *Paracentrotus* allowed to develop in acidified sea water show either a very slow development or a regression of the spicules, depending on the *p*H (Bouxin, 1926). Regression takes place below a *p*H of 6·4, and slowing up of the development of the spicules and of the larvae themselves below a *p*H of 7·2.

In adult echinoderms, the calcareous plates are mesodermal in position, but their formation has apparently not been studied from the cytological and physiological standpoints.

(8) *General conclusions regarding invertebrate skeletons*

The calcium required for skeletons is obtained either directly by absorption from the external medium or indirectly by the ingestion of plant or animal food. Direct absorption of calcium is possible in most and perhaps all aquatic invertebrates, and is probably the more important of the two sources of this element for skeleton formation. This absorption may take place only at well-defined intervals in the life cycle, as is the case in Crustacea. In American oysters it occurs apparently throughout the year, even at a temperature below 4° C., when the ciliary feeding mechanism is practically out of action, but the growth rings on the shells of temperate zone molluscs indicate that in these cases it is partly a seasonal activity depending in some way on temperature. The general contrast between the tropical zone with high temperatures (about 25–32° C.) and abundant calcium carbonate secreting organisms including corals, and the polar zones with a temperature approaching zero all the year round and but few organisms forming calcareous shells, is well known (Murray, 1908). But analysis of the relative importance of physiological and environmental factors responsible for this distribution has not been made.

The mechanism of the absorption of calcium ions is practically unknown, and in most cases it has not been settled whether the chief absorbing surface is the gut or the more permeable parts of the external boundary membranes. In the marine decapod Crustacea, simple diffusion through the soft integument and the gills probably plays a significant part in the absorption, since the normal adverse concentration gradient between the blood calcium and that of the sea water is reversed when crabs and lobsters have moulted and are absorbing calcium ions (Robertson, 1937, 1939, and unpublished data).

The carbonate radicle is probably in all cases derived from the animal's metabolic carbon dioxide, and so this excretory substance is being put to valuable use. How much of the normal output of metabolic carbon dioxide is fixed in this way is difficult to estimate, no data being available, but in animals where the amount of calcareous secretion is large in proportion to the mass of living tissue (e.g. sponge and echinoderm larvae), probably most of it is used, and additional carbon dioxide is absorbed from the surrounding water.

Skeletons as the name implies are chiefly supporting structures, and in a fairly constant environment such as sea water the thickness and strength of calcareous skeletons is approximately the same for members of a species. The variation of these characters with availability of calcium compounds or ions (e.g. molluscs, Foraminifera) indicates the automatic response of the secreting tissues to external conditions. Shells may be regarded as being formed to a certain extent of excretory material. In those terrestrial gasteropods in which some chalk is absorbed with the food, the chalk will be absorbed into the blood stream and then excreted into the shell, forming the chemically less active crystalline shell substance.

Exoskeletons when calcified, besides having a protective function, are relatively efficient barriers to the free diffusion of ions and to changes in osmotic pressure. For example, the soft uncalcified integument of Crustacea after moulting not only renders them comparatively helpless against enemies, but also decreases both their control over their salt content and their resistance to an adverse osmotic pressure (e.g. *Gammarus pulex*, Schumann, 1928).

The importance of calcium carbonate skeletons in maintaining the neutrality of the blood by buffering the acids produced in anaerobic metabolism is a feature primarily of molluscs. But any temporary acidity of water will be withstood better by animals with calcareous skeletons acting as buffer reserves than by soft-bodied animals (e.g. Kreps, 1929; Meyer, 1935).

IV. OTHER CALCAREOUS SECRETIONS

In most terrestrial oligochaetes there are present in the alimentary canal calciferous glands, which produce small spherules and larger crystalline concretions of calcium carbonate. The glands are specializations of the oesophageal epithelium, and the spherules of vaterite are formed inside the syncytial layers which form the gland lamellae (Harrington, 1899; Massal, 1929).

Recent surveys of the many functions ascribed to the glands are given by Dotterweich & Franke (1936) and Robertson (1936). The latter investigated the principal hypotheses. Feeding experiments carried out in earthworms by allowing them to ingest and absorb various calcium salts such as the phosphate, sulphate, carbonate, chloride and nitrate, established that the calcium of the secretion could be derived from any of these salts. Since in worms kept for example in moistened calcium sulphate the excretion of calcium carbonate continued indefinitely (8 weeks in one instance), it is obvious that in the intermediary metabolism of calcium the sulphate radicle is replaced by the carbonate radicle which must have come ultimately from metabolic carbon dioxide. In controlled experiments in which both the respiratory carbon dioxide and the carbon dioxide in the carbonate excretion were determined, it was found that the amount of carbon dioxide in the calcium carbonate varied between 3 and 9% of the total metabolic carbon dioxide, the average amount being about 5%. With leaves as the source of calcium, the figure was below this average.

It has long been imagined that the secretion will neutralize any acids present in the soil ingested and will bring the intestine to the most favourable reaction for the activity of its enzymes. This idea has arisen because the proteinase of the gut seems to be most efficient in an alkaline medium, and because the histological appearance of the glands after keeping worms in acid media such as lemon juice, starch and sawdust, suggests that they have been "over-secreting" (Harrington, 1899). If one compares the *p*H of ingested soil with the *p*H of the worm-cast, one finds there is a slight tendency towards neutralization, but this is due to the gut secretions other than the secretion of the glands. The hydrogen-ion concentration of soil of *p*H 7·7 and 6·6 increases after ingestion by earthworms, despite the fact that the vaterite spherules in the glands give an alkaline value of *p*H 9·4. The key to this result lies in the fact that the vaterite spherules coalesce and crystallize out as calcite concretions, the chemically inactive form in which the calcium carbonate escapes into the oesophagus. Earthworms form these concretions which are of no value for neutralization whether kept in alkaline or acid soil, or feeding on decaying leaves.

The real importance of the glands is the excretion of excess calcium salts in the form of calcite crystals which are relatively chemically inactive in the acidities encountered by earthworms. Members of the family Lumbricidae have been found by me in soils varying from *p*H 4·8 to 8·3, but they are most abundant in neutral soils.

Under exceptional conditions such as a high sustained carbon dioxide content of the soil atmosphere, the calcium carbonate spherules of the oesophageal glands will go into solution as the bicarbonate, thus acting as a buffer reserve in the maintenance of the reaction of the body fluids (Dotterweich, 1933).

Several secretions of practically unknown significance occur in the Platyhelminthes. In *Rhynchodemus*, a terrestrial triclad, small masses of granular slime are periodically excreted and left behind in the slime track. The granules are rhombic crystals of calcium carbonate (Percival, 1925) produced by ectodermal and subectodermal gland cells (Eastham, 1933). The presence of calcium carbonate in the mucus recalls the same occurrence in *Helix* (Prenant, 1924), and probably it should be regarded as an excretion of excess calcium absorbed with the food, comparable to the similar excretion in the other terrestrial group, the earthworms.

The calcareous corpuscles (calcospherites) present in the parenchyma of cestodes and in the excretory tubules of trematodes are also structures whose function has not been established. Those in the parenchyma of cestodes are formed inside connective tissue cells (Benham, 1901). The corpuscles may simply be excretory, being derived from excess calcium absorbed from the surrounding medium, and metabolic carbon dioxide, precipitated as the carbonate salt. Any acids produced during temporary anaerobic conditions to which these parasites must occasionally be exposed would be buffered by the solution of the calcium carbonate (Burian, 1924, pp. 401–4).

Calcium phosphate is present in cells of the digestive gland of gasteropods. The calcium may be utilized in shell regeneration as in *Helix* (Sioli, 1935), but here also the cells secrete rhythmically during feeding or starvation, passing the calcium phosphate spherules into the crop. Krijgsman (1928) has suggested without definite evidence that the spherules act as a buffer reserve regulating the *p*H of the crop.

V. IMPORTANCE OF CALCIUM IN RELATION TO ANIMAL DISTRIBUTION

That the distribution and abundance of some animals and plants can be correlated with the calcium content of the waters they inhabit is certain. But uncertainty and incomplete knowledge often exist as to the reasons for these correlations.

The concentration of calcium *per se* may often be important, especially to such animals as Foraminifera, molluscs, crustaceans and vertebrates which require it for their skeletons in addition to their requirements for normal metabolism. The existence and distribution of various soft-bodied invertebrates, such as the estuarine *Procerodes ulvae* and *Nereis diversicolor*, and freshwater planarians and worms, is governed by the concentration of calcium in the aquatic medium (see § II, (1) and (2)). But concentration of calcium is often associated with other factors. In peaty acid waters, for example, the general scarcity of organisms may be due to the interaction of at least three factors: acidity, deleterious effect of the humic acids, and lack of calcium. If these waters were mixed with water from a chalk stream, the increased calcium content would be associated with slight alkalinity and a neutralization of the effects of the vegetable toxins on animals. Few experiments have yet been done to assess the relative importance to animals and plants of these three factors. One such experiment concerned the absence of *Anopheles* larvae in peaty waters. At first this was thought to be due to the low calcium content of these waters, since females preferred waters richer in calcium for egg-laying (Smorodintzew & Adowa, 1928), but later it was shown that the primary factor was *p*H and not calcium content (Smorodintzew & Adowa, 1930*a*, *b*). *Anopheles maculipennis* larvae developed normally in *Sphagnum* waters and other waters of calcium concentration 10–73 mg. per litre provided that the *p*H was within or adjusted to be within the range 5–10.

Calcium may also be of importance indirectly by favouring the growth of particular food plants, and by causing the agglutination and precipitation of clay particles which would otherwise hinder light penetration and cause mechanical obstruction to members of the fauna.

The distribution of freshwater molluscs has been perhaps the most fully studied in relation to the calcium content of their environment (Boycott, 1936). To take a few examples, such molluscs as *Anodonta cygnea*, *Limnaea stagnalis*, and *L. auricularia* are found fairly generally in the south of England, but they are practically absent from Wales, the Lake District, and the south-west and the north of Scotland, a distribution correlated with the high calcium content of the waters they inhabit. Other species such as the universal *Ancylus fluviatilis* and *Limnaea pereger* are predominant molluscs in soft waters, but they can live almost equally well in hard waters in which they form much heavier shells.

The freshwater crayfish of Britain is absent from the rivers of the north of England and from Scotland, where the waters derived mainly from areas of igneous rocks have low calcium values. In a district in France (Sussac, Haute-Vienne) the occurrence of crayfishes in isolated areas in a predominantly granite country is associated with the presence of amphibolite, a hornblendic rock rich in calcium (Couégnas, 1920). The distribution of crayfish is obviously related to the calcium requirement for calcification of the skeleton. *Gammarus pulex*, another freshwater crustacean, has a restricted distribution. It has been established that a calcium requirement of at least 5 mg. per litre is essential for this species. Post-moult individuals die with their integuments still soft unless this amount of calcium is present in the water. Associated with this calcium factor is *p*H, the optimum range being 7·2–7·8 (Schumann, 1928).

Certain dipterous larvae such as the psychodid *Pericoma* are covered with crystalline spines of calcium carbonate. These larvae are found only in calcium-rich springs (Krüper, 1930).

The reason why some freshwater sponges are intolerant to excess calcium is quite obscure. One species (*Spongilla ingloviformis*) which colonizes Wisconsin waters of 3 mg. calcium per litre or less would not live in natural water (<1 mg. calcium per litre) to which calcium bicarbonate was added. Other species seem to have definite ranges of calcium which if exceeded experimentally cause their death (Jewell, 1939). In Ireland, Stephens (1920) has found that sponges are less numerous in lakes on limestone areas than in those on non-calcareous areas. *Heteromeyenia ryderi* was never found in limestone lakes. But the problem of sponge distribution here appears to be partly one of suitable habitats: the thick, soft, calcareous deposits on many of the stones in limestone lakes are unsuitable as a substratum for the growth of sponges.

The presence of calcium carbonate in the soil as chalk, limestone, or calcareous sand is an important factor in the distribution and abundance of land molluscs (Boycott, 1934). *Cyclostoma elegans* is found only on highly calcareous soil. At least eight other species, one of which is *Helix pomatia*, are cited by Boycott as obligatory calcicoles. Many others prefer calcareous habitats, and have thicker shells where there is an abundant source of calcium carbonate (see also §III, p. 119). The species indifferent to the presence of this compound are widely distributed, small and thin-shelled, and include the slugs with their much reduced shell.

Probably the main reason why snails are found chiefly in calcareous habitats is simply the need for calcium for shell formation. The thicker the shell the more protection will the snail derive from it. Limestone, with its fissures and recesses, also forms a more favourable situation for many species than igneous rocks.

VI. SUMMARY

1. In estuarine organisms a certain concentration of calcium in the external medium is essential for the control of permeability.

2. Calcium stabilizes intercellular matrices and mucous coverings. In its absence the constituent cells of most invertebrate tissues fall apart, and Protozoa and planarians lose their power of locomotion, since they are unable to adhere to the substratum.

3. Calcium is essential for the normal activity of the neuromuscular system, for amoeboid and ciliary movement, for chromatophore activity, and for the clotting of the body fluids of certain invertebrates. Lack of calcium causes a cessation of all rhythmical movements, loss of muscular tone and flaccid paralysis. Increase of calcium above the normal level leads to an acceleration of rhythmical movements, increase in excitability, and finally spastic paralysis. The effects of decrease and increase of calcium on the neuromuscular system in invertebrates are thus the opposite of those found in vertebrates.

4. Calcium carbonate for the formation of invertebrate skeletons is accumulated in the interior of cells or syncytia in an amorphous form or as vaterite spherules. In animals with non-spicular skeletons, the calcium carbonate is then released, and crystallization to form the skeleton substance takes place outside the cells.

5. Direct uptake of calcium from the external medium for the formation of skeletons is possible in most aquatic invertebrates, but the mechanism of absorption is practically unknown. In the marine decapod Crustacea immediately after moulting simple diffusion through the soft integument or the gills may take place, since the concentration gradient between the sea water and blood calcium is favourable at this period. The carbonate radicle of skeletons is derived primarily from the animal's metabolic carbon dioxide.

6. Skeletons function as supporting structures. Exoskeletons, besides having a protective function, are relatively efficient barriers to diffusion of water and ions, and in the Mollusca they often act as reserves which buffer the acids produced during periods of anaerobic metabolism.

7. The calcium carbonate formed by the calciferous glands of earthworms, and the crystals and vaterite spherules found in the mucus of terrestrial triclads and snails are probably to be regarded mainly as excretory material, since calcium compounds are usually abundant in the food or ingested soil of these animals and they seem to have no control over absorption.

8. The distribution and abundance of many aquatic invertebrates are correlated with the calcium content of the waters they inhabit. The reasons for these correlations are not always clear. However, the concentration of calcium *per se* is often

important especially to those animals which require it for calcareous skeletons and for the maintenance of control over permeability. Thus, for example, *Gammarus pulex* is unable to harden its integument after moulting unless the calcium concentration in the water exceeds 5 mg. per litre, and *Procerodes ulvae* is not found in waters with less than this amount of calcium. Most snails prefer calcareous habitats, and the species indifferent to the presence of chalk, limestone, or calcareous sand are usually small and thin-shelled. The latter include the slugs with their reduced shell.

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THE DISPERSAL OF THE ARTIODACTYLA

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No comprehensive account of the dispersal of the Mammalia from the Tertiary upward has been published since the appearance of Matthew's *Climate and Evolution* in 1915.[1] During the twenty-five years which have elapsed, many fresh facts have accumulated, and deductions which bear on the subject have repeatedly been made from them. Year by year new discoveries tend to clarify or alter our views, and it has seemed that the time is ripe to attempt a general synthesis of these facts and theories, with the hope that some approximation to the truth may emerge from it.

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[1] A revised edition of this, with trifling alterations by E. H. Colbert, has recently been published.

In the present article a beginning is made with the large order of the Artiodactyla, which seems to present a larger number of ascertained and well-authenticated data on which to found our conclusions than in the case of many other orders.

I. GENERAL PRINCIPLES

Any account of the dispersal of a biological group must of necessity start from the first differentiation of such a group and the knowledge, whether direct or inferred, of the region where that differentiation occurred. According to the most generally accepted view, the evolution of the group continued in that region and dispersal into other parts of the world proceeded as conditions of subsistence or climate compelled it. Often these emigrants must have developed secondary branches in areas remote from that in which the primary stock originated, and the history of these various branches up to the present day, or of their extermination either partial or complete at intervening epochs, would constitute a full record of the dispersal of the group.

It becomes more and more difficult as we go back in time to ascertain the facts on which such a record must be based. There are several reasons for this. One of the most important is that the problem of deciding on the affinity of any fossil remains is increased as we approach the point from which different lines diverged. Even complete mammalian skeletons may sometimes provide no sure evidence for their affinity. The almost completely known L. Oligocene genus *Leptomeryx* may serve as a case in point. While Prof. W. B. Scott includes this definitely among the Tylopoda (camels), the late Prof. W. D. Matthew regarded it as ancestral to the Cervidae (deer). There must be still less certainty when we know a mammal only by one part of the anatomy. Even from the teeth, which so generally furnish evidence of the ordinal position, it is often impossible in the absence of other parts of the skeleton to determine whether a primitive mammal is a condylarth, a primate, a carnivore or an artiodactyl. Another cause is connected with the apparent rapidity with which any striking differentiation is evolved from contemporary forms. This through the imperfection of the geological record minimizes our chance of finding connecting links. These difficulties apply, though in a less degree, to branches which arose late in the history of a group, as for example the Hippopotamidae and the Bovinae (buffaloes and oxen).

Our conclusions as to the origin and dispersal of the various mammalian groups must be based: (1) on the palaeontological record of each group as preserved in different parts of the world; (2) on the facilities for intermigration between one region and another which we can prove to have existed or on the contrary to have been impossible during different geological periods. The first of these is of very varying degrees of completeness in different regions, while the second, though theoretically ascertainable from the geological record, is in effect often inconclusive owing to the fragmentary nature of that record, so that in the main recourse must be had to the first.

Where a group appears suddenly in any particular part of the world without any apparent ancestors in the earlier deposits (assuming that they are adequately fossiliferous), we have a right to conclude that the group in question was immigrant. This inference has often been correctly drawn, although the region where the ancestral members of the group existed was at that time unknown. Thus the sudden appearance in the L. Miocene of Europe of well-differentiated proboscidean types which had no forerunners in the Oligocene showed that they entered Europe from without. It was not until much later that the occurrence of primitive Proboscidea in the U. Eocene and L. Oligocene of North Africa demonstrated that the order probably originated in Africa.

Matthew (1915, p. 201) has been at some pains to postulate that in the case of contemporary genera or species of any group, the more primitive are to be found in regions more remote from the centre of distribution and the more advanced nearer to it. This may be taken as a sound generalization, particularly in the case of living animals, though I cannot believe that it is of universal application. An emigrant species may sometimes have gone on developing in its adopted area, while it died out in its original homeland. No one will be disposed to deny that the centre of proboscidean evolution shifted from Africa to India in the Miocene, at any rate as regards the elephantoid group of that order, so that the most primitive forms of elephantoid might be expected to occur in India earlier than in Africa or elsewhere. Since we may assume that the time occupied by the migration of a species is geologically negligible in the absence of climatic or geographical barriers or other inhibitory conditions, it is quite unreasonable to suggest that if two closely related species at the same stage of evolution occur, one say in Europe and the other in India, one of the two is necessarily earlier than the other.

I will end this preamble by saying that to date the fossils referred to I have generally used the geological system names, since continental correlation can never be exact enough to enable us to fix a date by the smaller stage divisions so often employed. It is, however, necessary to add that contrary to the practice of many authors I include both the Pontian and Villafranchian stages in the Pliocene, and regard them as belonging to the lower and upper portions respectively of that system. I date the American faunas according to the correlation adopted in my paper of 1940*a*.

II. CENTRES OF MAMMALIAN EVOLUTION

Most authors who have discussed distribution problems connected with the Mammalia have adhered to the well-known zoological realms and regions instituted by P. L. Sclater and adopted with modifications by Wallace, Lydekker and Matthew. In assessing the value of the data which bear on the origin and dispersal of the Artiodactyla, I have chosen areas somewhat different from these, partly because it is evident that the Holarctic region cannot be regarded as uniform throughout on account of the alternating periods of isolation and connexion of Central Asia with Europe on the one hand and with North America on the other; partly because our knowledge is restricted by circumstances to smaller divisions of the earth's surface than those available for the study of living animals. These may or may not possess significance. Europe, Central Asia (including Mongolia and western Siberia), India, North Africa, Central and South Africa, North America, South America are the areas which have been considered individually. These areas differ greatly as to the richness and adequacy of their palaeontological record and as to their status as developmental centres of mammalian evolution. The geological succession is fairly complete in Europe, North America, and South America, but all the others contain numerous gaps. Europe possesses a vast number of mammal species scattered liberally throughout the Tertiary, though their remains are more fragmentary than in North America. It has, however, little or no indication of having been a great developmental centre as has probably been the case with each one of the others. Its fossil faunas seem to have been in the main immigrant, and although some of these immigrants have naturally evolved further in it, for example, the dichobunids, xiphodonts, dacrytheres, etc., yet no long-continued course of evolution can be proved for any European group such as we find in North America in the case of the Equidae, Brontotheriidae, Entelodontidae, Tayassuidae (peccaries), Oreodontidae, Camelidae, Merycodontidae, and Antilocapridae (prong-bucks); in Africa in the early Proboscidea and Hyracoidea; in India in the Anthracotheriidae, the elephantoid branch of the Proboscidea, the Boselaphinae and the Bovinae; in South America in the Notoungulata, Edentata and Liptoterna; and in Central Asia probably the Chalicotheroidea, Rhinocerotidae, Tragulidae, Cervidae and aegodont Bovidae. This may have been due to the frequent movements of sea and land in Europe and western Asia which prevented it from ever being a large, permanent land area suited for mammalian evolution. Europe in short seems to have been subject to repeated mammalian invasions throughout the Tertiary, few of the invaders having had a subsequent history except on a small scale.

It is true that we should not take it for granted that the absence of any particular group from one part of a large continent necessarily implies that it was equally absent from every part of it. Scott (1937) has repeatedly stressed the contrary as a possibility in suggesting that north-eastern America, which is for the most part barren of land deposits, may have originated groups which seem to be immigrant into the richly fossiliferous western and southern states. Such faunal isolation does not seem to me likely except on a small scale or for a short period, but one must not overlook the equal possibility that certain areas, whether large or small, in Central Asia or Africa may have harboured a mammalian fauna different from that which inhabited the remainder of the continent.

III. MIGRATION ROUTES

Despite the immensely important bearing which land connexions suitable for travel have on the problems of dispersal, we know little for certain as to when and how far each of the regions mentioned above was connected to or separated from the others by climatic or geographical barriers. We may perhaps set aside Wegener's theory of continental drift, since even if true it could not presumably have operated during the Tertiary period, with which alone I am now concerned. Still, apart from this, opinions on the subject range from authorities who have advocated land bridges of varying degrees of extension in time and space, connecting, for example, North America with Greenland and Scotland, South America to Africa across the Atlantic, Africa to India across the Indian Ocean, and even Asia to South America across the Pacific, down to those who contend for the permanence of the great ocean basins. Van Ihering, Scharff and Joleaud were among the best known authorities who adopted the former theory in its most extreme form,while Matthew (1915) accorded the most rigid support to the second. Personally, I am disposed in the main to accept Matthew's view and would invoke but few land connexions which do not exist to-day. Needless to say this does not apply to certain shallow-water straits in continental areas of which the most important are the shallow Behring Strait which now separates Alaska from Siberia, and land connexions from time to time in the Tertiary between Europe and Africa at various places now separated by the Mediterranean. Geological evidence in favour of these is incontrovertible. Geology can equally demonstrate that the sea has repeatedly transgressed over great portions of each one of the continents in Tertiary times. Such seas would obviously constitute an effective barrier to the migration of most land animals provided they were of sufficiently great extension to prevent animals from crossing them by taking a circuitous route. The narrow isthmus connecting North and South America can easily be shown to have entirely checked migration by land during the periods when the sea flowed over it. In many parts of the world, however, this proviso is impossible to prove with certainty through insufficient geological research, which time no doubt will remedy. For the most part geology neither definitely upholds nor contradicts the effectiveness of these barriers, any more than it can be said to do in the case of the majority of suggested land bridges, and we are driven to rely mainly on biological considerations.

It may, however, be worth while to refer as briefly as possible to the geological evidence which bears on the practicability or the reverse of routes which affect the migration of the Artiodactyla. It has generally been assumed by vertebrate palaeontologists that Central Asia afforded an open highway for land migrations between North America and Europe throughout the Tertiary. It is very doubtful if this is true. The continental tract extending from the Arctic east of the Urals to the Caspian and thence through western Persia to Arabia seems to have been most subject to marine transgression. Palaeocene seas seem to have existed in parts of this area (A. M. Davies, 1935, pp. 79, 80). Towards the end of the Palaeocene and during the L. Eocene a temporary recession of the sea affected large areas in eastern Russia (A. M. Davies, 1935, p. 90). The great marine transgression of the M. Eocene would appear to have submerged much of this area as it did the greater part of Persia, Asia Minor, North Africa and southern Europe. An emergence of land occurred in the U. Eocene and probably continued into the Oligocene. An Oligocene, perhaps U. Oligocene, sea washed the eastern flank of the Urals from the Arctic to the Caspian, and though this began to disappear in the L. Miocene simultaneously with the widespread emergence which took place in Europe, North Africa and Asia Minor, yet this sea, or the environment which its gradual desiccation produced, seems to have continued as more or less of a barrier to land migration up to the end of the Miocene (see Pilgrim, 1940*a*, pp. 3, 4). From then on intermigration between Asia and Europe seems to have been free.

An interesting paper accompanied by palaeogeographical maps was published by L. M. Davies (1940), in which it is clearly brought out that during the upper Palaeocene a relatively narrow isthmus separated the Tethys Sea from the Indian Ocean. This isthmus formed a link between Persia and the large land mass which included Arabia and most of Central Africa, probably extending to the Atlantic. We know of nothing against the supposition that this central African continent was connected with Europe at its north-western corner. In an easterly direction connexion with North America may have existed by way of the region lying just north of the site of the present Himalayas and China. India itself seems to have been a large peninsula adjoining the Tibetan portion of the route. In the L. Eocene, according to L. M. Davies, Africa and Arabia were cut off from Asia, and their separation became even more complete in the M. Eocene. The recession of the sea which began in the U. Eocene and continued in the L. Oligocene probably again opened a land connexion between India and Africa and with Europe through Asia Minor. As has been said above, there was an extensive land connexion between Europe, Africa, India and Central Asia during the L. Miocene, which accords with the homogeneity of the mammal fauna throughout most of this area. The marine transgression during the M. Miocene (see Pilgrim, 1940*a*, p. 5) is in harmony with the increasing isolation of the mammal faunas of the regions mentioned above. Towards the end of the Miocene land began to emerge and intercommunication became fully established in the Pontian (L. Pliocene).

Little evidence is available as to the influence of climate on migration during the Tertiary. Mountain masses offer almost an insuperable barrier to mammal migration mainly for climatic reasons. This factor has no doubt partly accounted for the relative isolation of India and the distinctness of its fauna during the latter part of the Tertiary. The north-east entrance to India, therefore, must have afforded the easiest route. The fact that the rest of India was for long periods entirely cut off by sea from other land areas must have contributed largely to this result.

It has generally been recognized that for obvious climatic reasons migrations must, other factors being equal, have been more rapid along lines of latitude than of longitude. During the Pleistocene, however, the rigours of climate in the Glacial period exterminated many of the Artiodactyla in temperate latitudes, as it did in the case of numerous genera belonging to most other orders, and has led to their survival at the present day in the equatorial tracts of Africa, America and the Oriental region.

IV. THE RADIATIVE CENTRE OF THE ARTIODACTYLA

It is beyond the scope of this review to discuss the origin of the Artiodactyla. Suffice it to say that some authorities (Matthew, 1915, p. 257) consider that a derivation from one of the Condylarthra is the most probable, though the earliest known Artiodactyla are too specialized to be immediately derived from the known Condylarthra, and this would imply that their divergence dates back to the Cretaceous. Pearson (1927, pp. 453–6) has shown that the otic region in *Pleuraspidotherium*, a supposed Condylarth from the U. Palaeocene of Cernay, forms a good starting point for the morphological characters of that part of the skull as displayed in the earliest Artiodactyla. Gregory (1910, p. 403), on the contrary, is inclined to favour a descent from the primitive mesonychid-arctocyonid creodont stock.

The earliest Artiodactyla definitely known are of L. Eocene age. These are *Diacodexis* in North America, from the Gray Bull (= Sparnacian), and *Protodichobune* in Europe from a horizon at Epernay which Teilhard (1916–21, pp. 67, 72) considers to be Ypresian (see also Stehlin, 1906, p. 668) and so apparently somewhat later than that of *Diacodexis*. The two genera seem to be related and most authors, following Stehlin (1906, p, 673), have placed them in the family Dichobunidae. Various lineages, *Dichobune*, *Meniscodon*, *Mouillacitherium*, and *Metriotherium*, represent the family in Europe up to the M. Oligocene (Stehlin, 1906, p. 670). *Wasatchia*, *Homacodon*, *Helohyus*, *Lophiohyus* and several other North American genera have been similarly classified by Sinclair (1914). The family or even the ordinal position of these is not always certain, but provisionally they may be placed in a family Helohyidae, which is probably not separable from the Dichobunidae (see Pilgrim, 1940*b*, p. 146). These forms carry the family on into the Bridger (M. Eocene), while *Gobiohyus*, Matthew & Granger (1925, p. 7), from the Irdin Manha formation of Mongolia further extends its range into the U. Eocene. Since neither the Palaeocene of North America nor Europe show any trace of allied forms, they are apparently invaders into both regions. Far from this being an isolated instance, it has long been recognized that the L. Eocene faunas of Europe and North America are almost identical. With but few exceptions the genera are the same and differences are merely specific. In a very much less degree this is true of the U. Palaeocene of the two continents. Simpson, who has reviewed the faunas of this period in a valuable article published in 1937,[1] remarks (p. 36) that the Cernaysian (U. Palaeocene) families of Europe are also present in the American U. Palaeocene, though except for *Plesiadapis* the genera are different.

Chiefly from the evidence in North America, Simpson (pp. 39, 42, 43) infers that the archaic mammals, which were of world-wide distribution in the Cretaceous, underwent a primary differentiation into partially modernized stocks and a secondary differentiation in the same part of the world into more advanced and still more modern forms, related to existing orders or even families. Since both of these faunas were invaders into North America and Europe, the inevitable conclusion is that these continents received their modernized mammals from a common source in so to speak two instalments, the first in the L. and M. Palaeocene and the second in the L. Eocene. The question now arises as to where this common source was located. Central Asia has for long been regarded by most authorities as the most probable centre of mammalian evolution. It is believed by them that other parts of the world, at least those that were accessible, received their mammal faunas by emigration from thence. When the Gashato fauna of U. Palaeocene age was discovered in Mongolia in 1923, it was confidently anticipated that it would prove to contain the ancestors of the L. Eocene mammal faunas of North America and Europe, and so confirm the theory. This anticipation has not been fulfilled (see Matthew *et al.* 1925, 1928, 1929). The Gashato fauna is approximately dated by the affinity between *Prodinoceras*, a primitive uintathere, and *Probathyopsis* of the Clark Fork (U. Palaeocene) (a more advanced species occurs in the L. Eocene), and between *Palaestylops*, a primitive notoungulate, and *Arctostylops* of the Gray Bull. These and others, among them being multituberculates, are essentially of an archaic type, though they are advanced and peculiarly aberrant. No modernized types are present. The natural inference is that we have here an archaic fauna which had become specialized by isolation. It is, of course, possible, as Simpson (1937, p. 39) suggests, that the truly ancestral L. Eocene mammals were living at the same time in Asia, but in a different environment. I find it, however, difficult to believe that if Central Asia as a whole contained mammals ancestral to the various L. Eocene groups of North America and Europe, the Gashato fauna would not show some trace of them.

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[1] *Biological Reviews.*

This is not the only argument against the radiative centre of the primitive Artiodactyla being in Central Asia. There is a strong presumption that not only were the amastoid groups of the Anthracotheriidae, Cebochoeridae, Choeropotamidae, Entelodontidae and Suoidea derived from primitive Dichobunidae (see Pearson, 1927, p. 457; Matthew, 1934, p. 208; Colbert, 1938*a*, p. 105; Pilgrim, 1940*b*, p. 146), but also the numerous Eocene and Oligocene mastoid selenodont groups of the Caenotheriidae, Xiphodontidae, Amphimerycidae, Anoplotheriidae and others, or at any rate had a common ancestry with them. Stehlin (1906–10) has repeatedly emphasized that none of these groups was endemic in Europe, but arrived there at intervals from a radiative centre which he supposes to have been Asia. Central Asia has by no means a continuous palaeontological record throughout the Eocene, Oligocene and Miocene, but at any rate certain epochs have furnished an adequate mammal fauna, such as the Irdin Manha and Shara Murun for the U. Eocene; the Shantung faunas described by Zdansky (1930) for the U. Eocene and L. Oligocene; the Ardyn Gobo and Hsanda Gol for the Middle Oligocene; the Betpakdala beds of western Siberia,[1] the *Indricotherium* and *Brachypotherium* beds of Turgai, of U. Oligocene and L. Miocene age respectively; and the M. (or U.) Miocene beds of Shantung and Tung Gur. It is a striking fact that none of these deposits contain any representatives of the European mastoid families mentioned except an astragalus from the U. Eocene of Honan questionably referred by Zdansky (1930, p. 25) to one of the Anoplotheriidae. Dichobunidae or Helohyidae also occur in U. Eocene beds (Matthew & Granger, 1925, p. 7; Zdansky, 1930, p. 28). Of the amastoid group Anthracotheriidae are found both in the Upper Eocene of Honan and Shansi (Zdansky, 1930, p. 14) as well as in the U. Oligocene and L. Miocene of western Siberia. On the other hand, Cebochoeridae and Choeropotamidae have not been found in Central Asia. A fragmentary upper tooth from the Irdin Manha has been questionably referred to the Achaenodontidae. Suoidea are unknown before the M. or U. Miocene, although both their branches, the Tayassuidae (peccaries) and the Suidae (pigs), appear in Europe in the L. or M. Oligocene, and in North America the first of these in the M. Oligocene. On the contrary, they abound in India from the L. Miocene onward, but the absence of Oligocene deposits does not allow us to predicate their existence previous to then. In Africa deposits previous to the Miocene are equally lacking, but Suoidea occur in the L. Miocene both in Southern and Central Africa (Stromer, 1926, p. 113). It is difficult to reconcile this lack of the early Artiodactyla with the idea that Central Asia was the radiative centre of the order.

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[1] The fauna of this deposit has been briefly mentioned by Flerov (1938, p. 94), but has not as yet been fully described.

Of recent years Matthew has been the main exponent of this theory (1915, pp. 174–9; 1930, pp. 36–42), which is primarily based on the supposition that the adverse environment of northern regions during the zonar periods of geological history when the land was uplifted and extreme limits of climate prevailed, would favour the evolution of new and progressive animal types in polar regions and the retreat of ancient and backward types southward. Schwarz, in a suggestive paper published in 1924, has opposed this view and has claimed that Africa was the chief developmental centre of the mammals. Matthew's arguments do not seem to me to be conclusive, and although I by no means go the whole way with Schwarz, I think there is something to be said in favour of the permanence of a fauna which developed slowly and steadily in the equatorial forests of the south, as opposed to the quickly adapted and specialized types of northern latitudes, which might not be able to resist climatic changes or compete with less specialized southern types. Those who champion Matthew's hypothesis cannot deny that the Proboscidea and Hyracoidea almost certainly originated in Africa, but that the former group was able subsequently to become dominant in northern latitudes. If this happened in one case, why should it not have happened in others, the actual evidence for which is unfortunately not available to us at present?

Although Simpson (1937, p. 30) regards the Palaeocene and L. Eocene mammal faunas of Europe and North America as immigrant into the areas where they are found, he hazards no opinion as to whence they came, beyond the probability that it was from a large land mass which had been separated from North America and Europe for long previous to the Palaeocene. He states that he could surmise such an area in any of several regions of the earth. He, however, leaves it as unknown and beyond profitable speculation. I think myself that it might be worth while to examine some alternatives to the Central Asiatic hypothesis. South America need not detain us, since even in the U. Palaeocene isolation from North America had produced a high degree of differentiation in its mammals. In later periods these archaic mammals continued their development apart and no faunal interpenetration between the two Americas occurred until the end of the Miocene (see Scott, 1928).

India might conceivably have furnished modernized Palaeocene mammal types both to Europe and North America, but the free connexion with Europe could not have continued for long after the end of the Palaeocene, if we accept L. M. Davies's views. India, therefore, could hardly have been the source of the L. and M. Eocene invasions which gave Europe its cebochaeres, choeropotamids, caenotheres, dacrytheres and others. By this process of elimination we arrive at what seems a plausible conclusion, that the modernized mammal fauna of the world became differentiated from some elements of the archaic stock during the Mesozoic in Central Africa. In the L. and M. Palaeocene this fauna began to invade other parts of the world. In the L. Eocene migration must have been sufficiently active to lead to the dissemination of practically identical mammal faunas into such widely separate regions as North America and Europe.

Beyond the regrettable fact that Central Africa supplies us with no fossil evidence in support of the theory, there is one serious objection to it. The Fayum in North Africa contains ossiferous deposits belonging to the U. Eocene and L. Oligocene (Andrews, 1906; Osborn, 1908; Schlosser, 1912; Schmidt, 1913). The first of these bears no relation to the contemporary faunas of the rest of the world and the second but a slight one. Primitive Proboscidea (*Moeritherium*), Hyracoidea, Barypoda (*Barytherium*) and Embrithopoda (*Arsinoïtherium*) form the bulk of the fauna. They are believed to be related to the Amblypoda and to have been derived from an ancient protungulate stock. In the upper beds primates, creodonts, insectivores and anthracotheres are met with, but no forms related to the Perissodactyla or Artiodactyla of Europe occur. The situation seems to bear a distinct analogy to that of the U. Palaeocene Gashato fauna. Both contain specialized archaic types in spite of the fact that *ex hypothesi* a modernized fauna existed at the same time in areas but little distant in either case.

The geological information in the case of North Africa seems to provide us with a feasible explanation of the anomaly, which it does not in the case of the Chinese area. During the L. and M. Eocene and possibly before, a great marine transgression affected large parts of north-east Africa. The Fayum, during the periods when it was not covered by sea, and in any case an extensive area adjacent to it, were isolated from the rest of Africa. Communication between Central Africa and Europe must have taken place by way of Morocco and Spain, for which there is some geological evidence. When the sea retired in the U. Eocene, the unsubmerged parts of this large island peopled the hitherto submerged Fayum with the highly specialized descendants of the archaic Palaeocene fauna. In the L. Oligocene communication with the rest of Africa began to open up; the modern fauna began to filter through, chiefly anthracotheres, but also primates and creodonts. Finally, uniformity in the African fauna was gradually secured during the remainder of the Oligocene. This account is naturally hypothetical, but it is backed up by some geological fact and may serve provisionally to permit us to adhere to what seems to be at any rate a plausible working hypothesis—a Central African developmental centre for the modernized mammalian fauna of the world.

V. AMASTOID ARTIODACTYLA

Pearson (1927) has distinguished one group of artiodactyl families from the remainder by the possession of certain characters in the basicranial region of the skull which seem to be important and which are typified by the disappearance of the mastoid from the surface of the skull. This group, which she terms Amastoidea, is composed of the Anthracotheriidae, Cebochoeridae, Choeropotamidae, Suidae (pigs), Tayassuidae (peccaries), Entelodontidae, Mixtotheriidae, and Hippopotamidae. She considers that these were derived from an original mastoid group to which the earliest known Artiodactyla, the Dichobunidae of Europe and the Helohyidae of North America, probably one and the same family, belonged. This group, which retained the mastoid, continued to evolve on many diverse lines in different parts of the world and constitutes the majority of the surviving members of the order. Although Matthew (1929, 1934), Simpson (1931), and Colbert (1938, p. 103) have either expressly or implicitly rejected Pearson's classification on the basis of the otic region, yet they admit the close connexion between the amastoid families and the Dichobunidae (see also Pilgrim, 1940*b*, p. 145). It is true that Matthew unites mastoid families like the Anoplotheriidae and the Oreodontidae with the amastoid Anthracotheriidae, which, following Pearson, I believe to be wrong. The result, however, as it appears on Matthew's and Colbert's charts, does not materially affect the phylogeny of the order as I understand it, nor the evidence for their dispersal. This will now be considered family by family on the available data.

(1) *Anthracotheriidae*

The discovery in 1916 (Pilgrim & Cotter, 1916, p. 42) of a varied and abundant primitive anthracothere fauna in the U. Eocene Pondaung beds of Burma, probably Ludian but in any case no older than Bartonian, prepared us to find that S.E. Asia was the developmental centre of the family. The resemblances of one of the genera, *Anthracokeryx*, to *Gobiohyus*, an U. Eocene helohyid from Mongolia (Pilgrim, 1928, pp. 4, 19–27; Colbert, 1938*b*, p. 374), also distinctly suggested the derivation of the family from some helohyid. Both of these suppositions have been virtually confirmed by the recent discovery in the M. Eocene of India (Pilgrim, 1940*b*, pp. 128, 145) of a relatively large species, *Anthracobune pinfoldi*, which possesses many dental characters allied to those of the Helohyidae, while by its size and other dental characters it should clearly be classified with the Anthracotheriidae. Though showing many primitive features, it is much specialized and cannot be directly ancestral to any of the Pondaung anthracotheres. Side by side with this form a small artiodactyl, *Khirtharia*, occurs, which may be a helohyid but which, by its lower and more bunodont molar cusps, clearly differs from any known dichobunid or helohyid. The U. Eocene anthracotheroid genera of Burma include *Anthracohyus*, a primitive low cusped form of unknown affinity and *Anthracothema*, which is possibly ancestral to *Anthracotherium*, though showing resemblances to the Indian L. Miocene species referred to *Brachyodus*. *Anthracotherium* appears in the earliest Oligocene of Europe and in beds of approximately the same age in China (Zdansky, 1930, p. 17). It probably migrated into both regions from India. *Anthracokeryx*, the best known Indian U. Eocene genus, is more clearly ancestral to the American *Aepinacodon*, the North African *Bothriogenys* and the European *Ancodon*, all three of which appear in the L. Oligocene. The small conservative genera *Microbunodon* and *Microselenodon* which occur in the U. Oligocene of Europe, but persist in India into the M. Pliocene, are apparently derived from *Anthracokeryx*.

The origin of the small, short-jawed genera, *Haplobunodon*, *Lophiobunodon* and *Rhagatherium*, from the U. Eocene of Europe and of *Anthracosenex* from the L. Oligocene of China (Zdansky, 1930, p. 14) is still obscure. Pearson (1927, pp. 438, 457) has shown that *Haplobunodon* possesses a mastoid, and suggests that, if anthracotheroid at all, it came from a very early offshoot of the mastoid stock. No ancestral type to it has been found in the U. Eocene of Burma, so that its forerunners, if Indian, must have existed prior to the Pondaung period.

The absence of anthracotheres from the U. Eocene of Mongolia militates against the possibility that the family was Central Asiatic in origin. The Indian occurrences in general strongly suggest that it arose in or near India from some Palaeocene or L. Eocene dichobunid of a primitive type, which settled in that region in the course of a migration from an area which I can only surmise to have been Central Africa, in accordance with the theory which I have already suggested (p. 143).

The later history of the family in India is entirely in harmony with the idea that India was its main diffusion centre. The L. Miocene fauna of the Bugti Hills may be said to have been dominated by the Anthracotheriidae, whose remains both in number and variety are unequalled elsewhere (Pilgrim, 1912; Cooper, 1924). Here we witness the gradual transformation of the five-cusped into the four-cusped upper molar. From India this *Hemimeryx* type of anthracothere spread into Central Asia, where it occurs in the U. Oligocene of Turgai (Borissiak, 1923) and Betpakdala (Flerov, 1938); into East Africa, where Andrews (1914, p. 172) has recorded its presence, while Leaky, MacInnes and Hopwood collected similar remains, which are as yet undescribed, from the same region; and into North America as the L. Miocene genus *Arretotherium* Douglass (1905, p. 269). In the L. Pliocene of India *Merycopotamus* is the direct descendant of *Hemimeryx*. This genus persists into the U. Pliocene of India and is the last member of the family. It has been recorded from the Pliocene beds of Tunis (Boule, 1910, p. 812; Roman, 1931, p. 30), Java (Koenigswald, 1933, p. 29), and China (Young, 1932).

(2) *Cebochoeridae*

Originally considered as primitive Suidae, it was demonstrated by Stehlin (1899–1900, 1908) that these forms, all of which can apparently be included in the genera *Cebochoerus* and *Choeromorus*, are not merely entitled to family distinction, but have only a remote relationship to the Suidae or Tayassuidae. This was fully confirmed by Pearson (1927, p. 412) from a study of the basicranial region of the skull. Appearing in Europe in the U. Lutetian and absent from the Sannoisian, their range may be considered as M. and U. Eocene. The absence of directly ancestral forms in the L. Eocene strongly suggests that they were invaders from another region, which Stehlin (1910, p. 1159), in accordance with his usually expressed views, would place in Asia. The family have never been recorded out of Europe, but the failure to find them in the U. Eocene of India or Mongolia militates against this view. The U. Eocene of North Africa shows no trace of them, and the fauna of the remainder of Africa is unknown previously to the L. Miocene. However, as has been surmised above (p. 143), I consider Central Africa is the most likely centre of their development.

(3) *Mixtotheriidae*

This family is confined like the Cebochoeridae to the M. and U. Eocene, and like them is unknown out of Europe. Pearson (1927, pp. 427–33) has emphasized their affinity both to the Anthracotheriidae and the Cebochoeridae, all three families arising from a common early stem. This, in my opinion, will probably be found in Central Africa at an epoch not more remote than the U. Palaeocene.

(4) *Choeropotamidae*

This family is in the main U. Eocene, but persisted into the early Oligocene. It is not known with certainty out of Europe. Colbert, however (1938*a*, p. 100), rather tentatively suggests that *Brachyhyops* of the U. Eocene of Wyoming, U.S.A., and *Parahyus* of the M. Eocene of the same region might be aberrant choeropotamids. The former genus shows the skull characters perfectly, but the tooth pattern is indistinguishable; the latter is only known by the lower jaw. Scott (1937, p. 393) refers both of them to the Achaenodontine subfamily of the Entelodontidae. It has also been suggested that *Anthracohyus* of the U. Eocene of Burma (see p. 144) might be a choeropotamid. Pearson (1927, p. 435) considered that *Choeropotamus* may belong to an early offshoot of the anthracotheroid stock. The data at present available are too inconclusive to allow us to decide as to the region in which the family originated or as to the direction in which its dispersal took place.

(5) *Entelodontidae*

These are generally divided into two subfamilies, the Achaenodontinae and the Entelodontinae. The former of these is exclusively North American, so far as we know at present, and is M. and U. Eocene. The latter is both European as well as North American, appearing in both continents in the L. Oligocene and lasting on into the Miocene. The New Jersey M. Miocene genus *Ammodon* is the last of the family, noteworthy also as being one of the rare occurrences of Tertiary mammals in north-eastern America. The single European genus *Entelodon* is very close to *Archaeotherium* of North America. In the latter continent many genera occur, which have been reviewed by Peterson (1909). They appear to represent different phylogenetic lines, which are still rather obscure. No other part of the world has yielded remains which are with certainty referable to the family. A fragmentary upper molar from the Irdin Manha (U. Eocene) of Mongolia was doubtfully identified by Matthew & Granger (1925, p. 6) as achaenodont, and *Bugtitherium* from the L. Miocene of India (Pilgrim, 1908, p. 153) might possibly be a highly specialized member of it.[1]

The Achaenodontinae and the Entelodontinae are almost certainly related, but the former is not ancestral to the latter, since it has lost the front lower premolar, the face is short and the orbit is open behind. *Parahyus*, if more were known of its structure, might definitely prove to be achaenodontine and form a connecting link between *Achaenodon* and some early member of the Helohyidae (see above, p. 146). Scott's view (1937, p. 394) that the two branches of the family represent two separate invasions into North America seems to be more probable. The Entelodontidae seem to exhibit characters which ally them to one or the other of the amastoid families, but are not sufficiently close to any single one to lead one to suppose that they branched off from it. It is, therefore, in my opinion, more likely that they represent one evolutionary line of a complex and varied differentiation, which took place at an ancient date, L. Eocene or even earlier, and produced most of the amastoid families more or less simultaneously and in one part of the world. Definite proofs that this region may have been Central Africa are of course lacking at present, just as much as they are in the case of other artiodactyl families.

(6) *Suidae and Tayassuidae*

These two families, typified by the living pigs of the Old World and the peccaries of America, were already distinctly differentiated in the M. Oligocene of Europe. They are, however, related, and being both invaders their common ancestor must be sought in the Eocene of another region. Stehlin (1899–1900, p. 426; 1908, p. 749) was the first to suggest the affinity of the L. or M. Oligocene *Doliochoerus* from the Phosphorites du Quercy in France[2] to the peccaries, of which the earliest American ancestor appears in the Oreodon zone of the White River (M. Oligocene) as *Perchoerus*. Pearson (1927) confirmed this fully and further regarded *Choerotherium* of the M. Miocene as probably representing a collateral branch of the tayassuid stock.

The earliest member of the Suidae (pigs) yet known is *Propalaeochoerus*, which occurs in the M. Oligocene of Europe (Pearson, 1927, p. 403). This lineage after its arrival in Europe can be traced, apparently without interruption, through the U. Oligocene and L. Miocene *Palaeochoerus* into the M. Miocene *Hyotherium*, which persisted into the L. Pliocene as *H. palaeochoerus*. Another lineage, equally immigrant, is *Listriodon*, which arrived in the L. Miocene and did not become extinct until the L. Pliocene. *Conohyus* of the M. Miocene differs from *Hyotherium* mainly by its enlarged premolars, but is probably also an invading form. A large pig, *Microstonyx*, with small canines is typical of the L. Pliocene. The two latest lineages to appear in Europe were *Potamochoerus* of the M. Pliocene, belonging to the same genus as the African river hogs of to-day, and *Sus*, which includes the majority of modern pigs outside Central and South Africa, and which we find first in Europe in the U. Pliocene. Both these two last genera were believed by Stehlin (1899–1900, p. 151) to be directly descended from *Hyotherium*. The occurrence of forms close to *Potamochoerus* in the M. Miocene of India and of *Sus* but little later in the same region (Pilgrim, 1926) militates against this view, and we must regard them as also immigrants into Europe.

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[1] The incisor described and figured as that of this genus has since been shown to be the lower incisor of a baluchithere.

[2] Colbert (1933, p. 8) is probably mistaken in placing the horizon of *Doliochoerus* in the U. Oligocene (Aquitanian) (see Depéret, 1906, p. 618). *Doliochoerus* predates rather than postdates *Perchoerus*.

Lack of palaeontological evidence prevents us from deciding definitely as to where the diffusion centre of the Suidae was situated. Remains of pigs are not abundant in the Miocene and Pliocene of Africa. A form from the L. Miocene of South-West Africa has been referred by Stromer (1926, p. 114) to *Propalaeochoerus*, and a still more interesting form, *Diamantohyus*, occurs in the same beds (Stromer, 1926, p. 113). This genus is rather incompletely known and its affinities are obscure. The upper molars are four-cusped without a mesostyle; all the cusps are low; the inner cusps are crescentic, and the bunodont outer cusps have fore and aft crests ("Gipsnähte"[1]). P^3 is typically suid with two tall outer cusps and an inner cusp which is displaced to the rear of the tooth. The absence of a mesostyle precludes a reference to some four-cuspidate anthracotheroid and the shape of P^3 to a cebochoerid. The most likely supposition, in my opinion, is that *Diamantohyus* represents a survival of a stage of suid development which was more primitive than *Palaeochoerus*, a stage which Stehlin (1908, p. 748) predicated must have preceded the "neobunodont" Suidae. It may be recalled that *Propalaeochoerus* has indications of similar selenodonty in the inner cusps of the upper and the outer cusps of the lower molars. A small lower molar from Koru in Dr A. T. Hopwood's collection from Lake Victoria shows somewhat similar characters. Were it not for the scarcity of suid remains in African deposits, the occurrences just mentioned might induce us to consider Central Africa as the diffusion centre of which we are in search.

The abundance of Suidae in India in the Middle and Upper and probably throughout the Miocene arouses a suspicion that the developmental centre, even though not originally in India, may have shifted there or at any rate farther east than Central Africa during the Oligocene. The L. Miocene of India contains both the European genera *Palaeochoerus* and *Listriodon*. Their scarcity as compared with the abundance of Anthracotheriidae may only be due to their inability to face competition with the latter family in that particular environment rather than to an equal numerical inferiority in other parts of the same region. However that may be, from the M. Miocene up to the end of the Pliocene Suidae are a dominant element in the fauna (Pilgrim, 1926). *Palaeochoerus* persists up to the L. Pliocene. *Conohyus* is abundant and its premolars continue to develop on the same lines, passing into a stage represented by the Lower to U. Pliocene *Sivachoerus* (also recorded by Tobien (1936) from the M. Pliocene of Egypt), and culminating in the grotesque enlargement seen in the L. Pliocene *Tetraconodon*. *Listriodon* passes through stages identical with those of Europe and persists into the L. Pliocene. In the M. Miocene *Propotamochoerus* is represented by several species; another genus, also M. Miocene, but on a different line, *Dicoryphochoerus*, abounds. A true *Sus* appears at any rate in the U. Miocene and throughout the Pliocene branches into numerous species. Aberrant branches occur: a L. Miocene form which shows some affinity to the European *Xenochoerus*; *Sanitherium* of the M. Miocene; *Hyosus* and *Sivahyus* which culminated in *Hippohyus*, a very hypsodont form with complicated enamel folds. This did not become extinct until the U. Pliocene.

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[1] This expressive term has been used by Rutimeyer, Stehlin (1908, p. 691) and others.

As against this diverse evolution in India we have to note that Suidae are not recorded from any Oligocene or L. Miocene deposits in Central Asia. A *Listriodon* exists in the M. (or U.) Miocene beds of Tung Gur in Mongolia (Colbert, 1934), and from the L. Pliocene beds of China Pearson (1928) has described species of *Propotamochoerus*, *Microstonyx*, a large bunodont *Listriodon*[1] and *Chleuastochoerus*, which its author thinks is a derivative of *Palaeochoerus*. This fauna seems to have been derived partly direct from Europe and partly from India.[2]

Suidae, both living and fossil, are entirely absent from America. *Sus* spread apparently from India or some centre west of India into Europe, Central Asia, Malaysia (Java) and northern Africa. Colbert (1935*g*, pp. 257–60) has suggested that the U. Pliocene Indian species *Sus falconeri* would serve as a very satisfactory ancestor for the African wart hogs, *Phacochoerus*. These are represented in the Pleistocene of Africa by a great variety of genera and are at present entirely African, but their occurrence fossil in Palestine has been recorded by Bate (1934, p. 120).

The Tayassuidae seem to have reached North America in the M. Oligocene. No certain evidence as to their starting point or route can be adduced. A true, though somewhat aberrant peccary, *Pecarichoerus*, has been recorded from the M. Miocene of India (Colbert, 1933*g*). This is probably allied to the genus *Lophochoerus* Pilgrim (1926, p. 28), from Indian beds of the same age or slightly younger and to the contemporaneous European genus *Choerotherium* which Pearson (1927, p. 405) regards as an aberrant tayassuid. These occurrences suggest that the migration route lay through or near India, such forms being survivals from a primitive peccary stock.

A few words will suffice for the history of the peccaries in America, since they never left the New World after their first arrival there. The White River genus *Perchoerus* passes into the possibly congeneric *Thinohyus* of the John Day and *Desmathyus* of the Gering (L. Miocene). The latter genus seems to have been the common ancestor of three different lineages: (1) that of *Platygonus*, the most specialized, and very abundant in the Pleistocene; (2) that of *Prosthennops* and *Mylohyus* (neither of these two lineages outlasted the Pleistocene); (3) that leading through *Pediohyus* to the modern genus *Tayassu*. Some time in the Pliocene the latter genus spread to South America, which remains to-day almost the exclusive habitat of the family, which was virtually extinguished by the glacial period in North America, except in some of the southern states, such as Texas.

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[1] This species may belong to a different deposit from the rest of the fauna and may be as old as M. Miocene

[2] Pearson (1928, p. 74, footnote) suggests that some of the Indian specimens from Chinji or Dhok Pathan referred to *Palaeochoerus* may belong to *Chleuastochoerus*.

(7) *Hippopotamidae*

The earliest known members of this family, which appear in the L. Pliocene of India and but little later in Italy, are hardly separable except subgenerically from the living *Hippopotamus*. They may be assigned to *Hexaprotodon*. This genus is common in the M. and U. Pliocene of India and North Africa. In the Narbada beds (Pleistocene) of India both *Hexaprotodon* and *Hippopotamus* are represented. In the Pleistocene of Java, Central and North Africa, Palestine and Syria, hippopotami are fairly common, as they are in Europe. They vanished from Europe during the later Pleistocene, but may have persisted in India into historic times (see Pilgrim, 1925, p. 205).

The origin of the family remains obscure. The similarities to the anthracotheroid *Merycopotamus* were stressed by Lydekker (1876, p. 144) in a detailed description of the osteology of that genus. Other authors have advocated the derivation of *Hippopotamus* from some member of the Anthracotheriidae, the latest of them being Colbert (1935*d*, p. 10), who has made minute comparisons between *Hippopotamus* and other families and has decided in favour of an anthracotheroid origin. Stehlin, however (1908, p. 751), considered that the Eocene genus *Cebochoerus* was more likely to be ancestral to it. Pearson (1927, p. 435) accepted this and envisaged the unknown Oligocene hippopotami as lagging behind the contemporary Anthracotheriidae and following lines more nearly akin to the relatively conservative Cebochoeridae. One objection to this theory is that no matter how small a portion of the earth's surface was responsible for the differentiation of the hippopotami, it is inconceivable that they should have been confined to it from the Oligocene up to the L. Pliocene. Miocene beds in either Africa, Europe, India or Central Asia would surely have had some trace of them. If, on the other hand, the family arose relatively late in geological history, say in the M. Miocene, and in a very restricted area, as I believe to have been the case, an opinion also shared by Colbert so far as I can gather, then if its development were rapid, it would not be surprising to meet a fully fledged *Hippopotamus* over a wide area soon after. I have already (Pilgrim, 1925, p. 206) suggested Arabia as one such possible area. This is, of course, mere conjecture. Somewhat analogous developments have occurred in many groups. I need only mention that of the Bovinae in the U. Miocene. I consider that Colbert's observations on the anatomy of *Hippopotamus* and the tetracuspidate Anthracotheriidae respectively afford adequate support for the hypothesis. Its proof must await the discovery of the fossil fauna of the area in question.

VI. THE EARLY MASTOID SELENODONTS OF EUROPE

The Dichobunidae and their probable invasion of Europe and North America have already been discussed (p. 140). A brief mention of the other early mastoid selenodont families of Europe will suffice, for the reason that the occurrence of any of them outside Europe is exceedingly doubtful. Even in Europe their sojourn was relatively short and they were overwhelmed by later traguline and pecoran immigrants from Asia.

Different views as to the classification of many of the genera are held by, for example, Stehlin (1906–10), Schlosser (1923), Pearson (1927), Matthew (1929, 1934) and Simpson (1931). Without entire prejudice to other opinions, I accept the following families as coming into this group: Caenotheriidae, Anoplotheriidae, Xiphodontidae, Amphimerycidae, and Haplobunodontidae.[1] The only occurrences of any of these outside Europe, so far as I am aware, are that of an astragalus from the L. Oligocene of China referred by Zdansky (1930, p. 25) somewhat questionably to an anoplotherid, and of *Anthracosenex* considered by Zdansky to show affinities to *Haplobunodon*. All of these families come within the period embraced between the M. Eocene and the L. Miocene for the most part in the middle portion of it. They have been regarded by Stehlin as probably the result of separate immigrations into Europe. They seem to stand in no close relation to any of the early North American selenodont forms, though in both continents the genera in question possess characters and potential trends of development, which in each case they have probably inherited from the same or a similar primitive dichobunid stock. Matthew (1929, p. 407) has claimed that the Xiphodontidae are related to the North American Camelidae, but it seems questionable whether the precocious didactyly, the free cuboid and navicular, the length and advanced molarization of the premolars, the incisiform lower canine, the caniniform P_1 and the probably small upper canine imply close relationship in the absence of evidence of geographical proximity.

VII. THE EARLY MASTOID SELENODONTS (HYPERTRAGULIDAE) OF NORTH AMERICA

One striking distinction between the genera included in this section and the European ones which have just been considered is that whereas the latter have but a short history and are unconnected with any of the large later families, the former bear evident signs of being ancestral either to actually living families or to families which have a long, continuous later history. It is not always that universal agreement has been reached as to which family is descended from each of them, since they are often incompletely known and belong to a rather generalized type which might have preceded the differentiation into many recognized families. The name Hypertragulidae would almost seem to have been applied to the members of this group rather as a confession of ignorance than because the characters which they share in common possess any definite significance. As soon as certainty is acquired as to the phylogenetic position of a particular genus, there seems no reason to retain it in the Hypertragulidae. This procedure has been generally adopted in the case of genera like *Protoreodon*, *Protagriotherium*, *Protylopus*, *Blastomeryx* and *Leptomeryx*. The first three of these are included in the Oreodontidae, Agriotheriidae and Camelidae respectively, while Matthew (1908) considered the two last as ancestral to the Cervidae.[2] Many of them are U. Eocene. These include *Leptotragulus*, *Leptoreodon*, *Camelomeryx* and *Oromeryx*. *Hypisodus*, *Hypertragulus*, *Nanotragulus* and *Leptomeryx* are Oligocene and *Blastomeryx* Miocene. Scott (1937, pp. 306, 344) puts most of these genera, together with the curious, many-horned *Protoceras* and allied forms, into a family which he calls Leptomerycidae and includes them with the Camelidae and the Oreodontidae in the suborder Tylopoda. He does not indeed regard any of these genera as ancestral to the U. Eocene *Protylopus*, *Protoreodon* or *Protagriotherium*, but rather as cousins descended from unknown M. Eocene forms which may one day be discovered in the Bridger, or in contemporaneous beds elsewhere in America, or in Asia. Though admitting a remote connexion with the Helohyidae, he does not consider that any helohyid can be directly ancestral to the group. Proof of an Asiatic origin is lacking, since L. and M. Eocene deposits are so far unknown in Central Asia, but it seems feasible, though the failure to find allied forms in the U. Eocene of Mongolia is strange.[1]

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[1] This family is *incertae sedis* (see above, p. 145).

[2] Scott regards *Leptomeryx* as related to the Camelidae.

VIII. THE CAMELIDAE

The special characters of the modern *Camelus* can be traced back with certainty to *Poebrotherium* of the M. and U. Oligocene, less certainly but still with considerable probability to *Eotylopus* of the L. Oligocene and *Protylopus* of the U. Eocene. Four distinct lineages are recognizable in North America. Three of these were confined throughout their history to that continent, namely: (1) the giraffe camels beginning with *Paratylopus* in the Oligocene and passing through *Miolabis* and *Oxydactylus* to *Alticamelus*, which died out in the L. Pliocene; (2) the gazelle camels known by *Stenomylus* of the L. Miocene and *Rakomylus* of the L. Pliocene; (3) *Pseudolabis*, an Oligocene side line, of whose ancestry nothing is known. The fourth (4) can be traced back from the living Asiatic camel through the U. Miocene and L. Pliocene *Procamelus*, the M. Miocene *Protolabis* and the U. Oligocene *Protomeryx* to the M. Oligocene *Poebrotherium*. All these intermediate stages from *Procamelus* back are exclusively North American, as are also various Pliocene and Pleistocene species descended from *Procamelus*, of great size and more specialized than the living *Camelus*, which was not developed in America. The best known of these is *Camelops*, which persisted into recent times, as proved by the discovery in Utah of a skull with dried flesh still adhering to it (Romer, 1929, p. 261).

The Camelidae appear suddenly as invaders in the U. Pliocene of Asia. The widespread genus *Paracamelus* ranged from Kherson in eastern Europe (Pavlov, 1903, p. 118) to Choukoutien in eastern China. This is of much larger size than the modern camels, but appears to be approximately in the direct line of ascent to *Camelus* and is as nearly as possible the descendant of the American *Procamelus*. *Camelus* is represented by extinct species in the U. Pliocene of India (Falconer, 1868, i, p. 227), west Siberia (Orlov, 1929, p. 551) and Bessarabia (Pavlow, 1903, p. 113). Apparently it migrated back to America in the Pleistocene if its occurrence in Alaska recorded by Gidley in 1913 is correct. *Lama*, which is now exclusively South American, is also descended from *Procamelus* and migrated to South America in the Pliocene.

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[1] *Archaeomeryx* (see p. 152) is included by Matthew (1925) and Colbert (1941) in the Hypertragulidae.

IX. THE OREODONTIDAE AND THE AGRIOCHOERIDAE

These two closely allied families probably do not occur outside North America. From their appearance in the U. Eocene up to their extinction, that of the former family in the L. Pliocene and of the latter in the U. Oligocene, their development took place entirely in North America. Many authors (Schlosser, Matthew, Simpson) have thought that these families were related to the Anthracotheriidae. This opinion seems to be based mainly on the resemblance of the molars in the two. Scott (1937, p. 376) considers this resemblance as due to convergence, and remarks that as the Agriochoeridae are traced back in time they become less like the anthracotheres and more like the oreodonts, which would not be the case if they were nearly related to the former. If we accept Pearson's theory of the ancient divergence of the amastoid from the mastoid Artiodactyla, then the mastoid oreodonts clearly cannot be related to the anthracotheres, which branched off from the primitive stem very much earlier (see Pearson, 1927, p. 457). Scott includes both the oreodonts and the agriochoeres in the Tylopoda, but as is the case with all the U. Eocene forms belonging to that suborder, the direct ancestors have not been discovered. The Oreodontidae have developed along numerous lines, which has been well demonstrated by Thorpe (1937), but any account of these is beyond the scope of this review, as none of them left North America.

X. THE TRAGULINA

Matthew and Simpson have included these with the Pecora in a single suborder. Though both groups have a common ancestor, that ancestor is as far back as the U. Eocene. The living representatives of the Tragulina have retained many of the primitive characters which have been lost in all except the earliest Pecora. The latter, on the other hand, have developed in many remarkable directions quite remote from any traguline. These considerations urge me to keep the two suborders distinct. This view takes no account of the fact that the term Pecora, used by Linnaeus, has for long been restricted to the families which I now include in it, and any widening of its application seems to me unwarranted. Scott's opinion (1937, p. 305) is the same as my own.

The Mongolian genus *Archaeomeryx* from the Shara Murun U. Eocene is the earliest traguline of which we possess anything like a complete knowledge. This was briefly described by Matthew & Granger (1925, p. 9) and a more detailed study by Colbert is in the press. Among other characters, the fusion of the navicular and the cuboid, the somewhat enlarged, pointed upper canine, and the reduction of the fibula to proximal and distal vestiges distinguish it from the contemporary Camelidae and Oreodontidae. As Matthew remarks, "it lacks any of the various aberrant specializations which exclude all of the hitherto described Eocene genera from direct ancestry to the Pecora". Its primitive character is shown by the possession of a full incisor series, a primitive premolar structure, the freedom of the median metapodials and the retention of the lateral digits. Except the first, these characters have been retained in a greater or less degree by the modern tragulids, though in the Oligocene family Gelocidae the lateral metapodials have been almost lost. Although Matthew includes *Archaeomeryx* in the Hypertragulidae, yet we may, I think, fairly regard it as a primitive traguline. Another contemporary form, much less completely known but apparently traguline, occurs in the U. Eocene of Burma, *Indomeryx* Pilgrim (1928, p. 33). Since no similar forms occur in North America, there is a strong presumption that the Tragulina originated in the Eocene of Central Asia. The absence of a L. and M. Eocene fauna in Asia at present deprives us of the opportunity of finding a connecting link between these early Tragulina and the early Helohyidae, which are presumably their remote ancestors.

The dispersal of the Tragulina into other parts of the world began with the appearance of *Archaeomeryx*, if not earlier: to Europe in the U. Eocene if *Gelocus minor* Pavlow (1900, p. 40) really came from Mormont. In other parts of Europe *Gelocus* is common in the L. Oligocene of Ronzon and the Phosphorites du Quercy. According to Stehlin (1910, p. 1088) the Gelocidae should form a family distinct from the Tragulidae because of the reduction of the lateral metapodials which is greater than in the recent Tragulidae and the fusion of the median metatarsals to form a cannon bone, in which it agrees with *Tragulus* but differs from *Dorcatherium*. The navicular and cuboid, though fused, remain independent of cuneiforms II and III. In *Tragulus* and *Dorcatherium* cuneiform III is united with them. *Bachitherium* and *Prodremotherium*, common in the M. Oligocene of the Phosphorites du Quercy, are included by Schlosser in the Gelocidae. They have one premolar less than *Gelocus*, and P_4 is more advanced.[1] *Prodremotherium* seems to have persisted into the Upper Oligocene of Europe (Viret, 1929, p. 229). *Paragelocus scotti* Schlosser, apparently from the L. Oligocene of Germany, possesses a protoconule in the upper molars. According to Stehlin (1910, p. 1089) this is not a valid distinction from *Gelocus*, the upper molars of which also often show a protoconule. It would, however, seem to imply that the family branched off from the primitive tragulid stock earlier than *Archaeomeryx*, in which the upper molars are tetracuspid.

If we include *Archaeomeryx* in the Tragulidae, the next earliest occurrence of the family seems to be as *Lophiomeryx* of the M. Oligocene of Europe. A form from the Ardyn Gobo formation of Mongolia, which seems to be of approximately the same age, has been provisionally referred to the same genus (Matthew & Granger, 1925, p. 7). In *Lophiomeryx* the median metapodials are separate and the lateral metapodials are quite complete. In regard to the structure of P^3 it also seems to be more primitive than *Gelocus*. *Miomeryx*, also from the Ardyn Gobo of Mongolia, is an allied genus. In *Cryptomeryx* from the Phosphorites du Quercy, apparently contemporaneous with *Lophiomeryx*, we find the first indication of the folds on the posterior side of the anterior crescent of the lower molars, which is characteristic of the modern West African species *Dorcatherium aquaticum* (Schlosser, 1887, p. 93). The genus *Dorcatherium* occurs first in the L. Miocene of the Sables d'Orléanais (Stehlin, 1907, pp. 529, 531; Mayet, 1908, p. 128), but is much more common in the M. Miocene. It differs from the living species by little except the presence of P_1.

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[1] I can find no authority for the statement by Flower & Lydekker (1891, p. 307) that *Bachitherium* has traces of minute upper incisors.

Tragulids, which have not been definitely determined generically, have been recorded from the L. Miocene of India (Pilgrim, 1912, p. 76; Cooper, 1915, p. 407) and from South-West Africa (Hopwood, 1929, p. 5). In India *Dorcatherium* and an allied genus *Dorcabune* are common from the M. Miocene to the L. Pliocene and only died out in the M. Pliocene. No records of Tragulidae from the Miocene or Pliocene of Central Asia exist, but starting from its probable diffusion centre in that region, the family evidently wandered over the whole of the Old World, but has survived to-day only in *Tragulus* of India, Ceylon and Malaysia and *Dorcatherium* of West Africa.

XI. THE ANCESTRY OF THE PECORA

The probability that Central Asia was the developmental centre of the Pecora has been much increased by recent discoveries in that region, especially those which led Teilhard to suggest a giraffoid affinity for the Lower Miocene *Procervulus* of Europe and the L. and M. Miocene *Lagomeryx* of Europe and Mongolia. This will be rendered still more certain if *Aletomeryx* and *Dromomeryx* and their numerous allies from the L. and M. Miocene of North America prove also to be giraffoid but of a less advanced type, as I have suggested elsewhere (Pilgrim, 1941 *a*). I have supposed that the giraffid type of "horn" was nearest to the primitive stage, from which every other type of pecoran "horn" was derived, though perhaps no long period elapsed between each.

In any case it is obvious that each type of "horn", whether lagomerycid, giraffid, cervid or bovid, must have been preceded by a hornless stage. Numerous hornless pecoran genera are known: *Dremotherium* and *Amphitragulus* from the U. Oligocene of Europe; *Blastomeryx* from the L. Miocene of North America; *Palaeomeryx* from the L. and M. Miocene of Europe. Of these *Blastomeryx* is completely known, *Dremotherium* and *Amphitragulus* much less so and *Palaeomeryx* by little except the dentition. In the present state of our knowledge it is impossible to predicate that one of these more than another is ancestral to any particular pecoran family. *Blastomeryx* has been shown by Matthew (1908) to be an ideal ancestor for the American Cervidae, and it should be remembered that hornless deer, *Moschus* and *Hydropotes*, still exist in Central Asia. At the same time it seems difficult to deny that such a form as *Blastomeryx* may have given rise to the Lagomerycidae, Giraffidae or even the Bovidae. The wisest course, in my opinion, is provisionally to put all these hornless Pecora into a single family, Palaeomerycidae, which we may regard as consisting of generalized types which were differentiated into the various pecoran families.

Excluding *Leptomeryx*, which is much more primitive, and whose affinities are still uncertain, *Blastomeryx* is the earliest member of the Pecora so far known from America. Since it is predated in Europe by *Dremotherium* and *Amphitragulus*, both invaders, we must suppose that Central Asia was the radiative centre of the suborder as a whole. The Tragulina have been shown to have their origin in that region, and the U. Eocene *Archaeomeryx* and other Asiatic fossils provide a branching off point for the ancestral Pecora probably in the Oligocene (Matthew, 1925; Colbert, 1941). It need cause no surprise that intermediate stages have not so far been found, since the L. Miocene and Oligocene faunas of Central Asia are either unknown or scanty.

XII. THE GIRAFFOIDEA

(1) *Lagomerycidae*

Teilhard's (1939, p. 269) view that *Lagomeryx* and *Procervulus* are aberrant, or, as I should prefer to call them, precocious giraffoids has been elaborated in a recent paper by myself (Pilgrim, 1941 *a*). *Climacoceras* MacInnes (1936, p. 521) from the M. Miocene of Lake Victoria Nyanza having small, irregular branches from a non-deciduous stem seems to find a place in the same family. If I am right in regarding the American *Dromomeryx* and *Aletomeryx* as representative of the simple unbranched horn stage which must have been ancestral to genera like *Procervulus* and *Lagomeryx* with branched horns, then these American L. and M. Miocene forms must be survivals from Oligocene ancestors, since *Procervulus* is L. Miocene. They appear to be immigrant in America and could hardly have come from elsewhere than Central Asia.

The family has retained many primitive characters lost in the Giraffidae, among which are the tusk-shaped upper canine, the separate radius and ulna, and the vestigial lateral metapodials. If proof is forthcoming that "ossicones" existed in young stages of *Dromomeryx* and *Aletomeryx*, then this is also a primitive feature, which the Giraffidae have retained, but which is unrecognizable in the Cervidae and Bovidae, except microscopically in juvenile or foetal stages, on account of the hypertrophy of the frontal apophysis, which surrounds and obliterates all trace of the ossicone (*os cornu*) at a premature stage of growth. On the other hand, the Lagomerycidae foreshadow developmental trends found in the Giraffidae such as the backward extension of the supraoccipital, the triangular section of the "horn", the absence of a lachrymal fossa, and the molarization of the premolars. *Lagomeryx* and *Procervulus* have anticipated a complexity of horn development only attained much later in the sivatheres. The relation between the two giraffoid families seems to present some analogy to that which exists between the bovoid families of the Merycodontidae and the Antilocapridae (see Pilgrim, 1941 *a*, and below, p. 159).

(2) *Giraffidae*

The earliest giraffid remains so far definitely known are probably those from the M. (or U.) Miocene beds of Tung Gur (Mongolia) referred by Colbert (1936) to *Palaeotragus*. The four-horned *Giraffokeryx* (Pilgrim, 1911, p. 14; Colbert, 1933 *e*) from the Chinji stage of India is approximately of the same age. The exact classification of *Propalaeomeryx* (= *Progiraffa*) *exiguus* Pilgrim (1912, p. 77) from the L. Miocene of India is very doubtful. *Palaeotragus* did not reach Europe until later; it occurs at Sebastopol in the topmost Miocene. The same as well as larger, allied genera, of which *Samotherium* is the best known, have been found in the L. Pliocene of Europe, Persia and China. All these forms are already much more progressive than the contemporary *Aletomeryx*, *Dromomeryx* and other Lagomerycidae, except in the complication and perhaps premature ankylosis of the "ossicones" in the latter family. It follows, therefore, that some considerable period must have separated these early Giraffidae from the common ancestor of both families. No such ancestral form is present in the L. Miocene faunas of Europe, India and America, with which we are well acquainted. On the other hand, since faunas of that age are unknown in Central Asia, the latter region is the most likely source of the radiation. This would be in harmony with the general ruminant differentiation which seems to have taken place in that part of the world.

In the L. Pliocene the true giraffine type appears. The widespread distribution of this subfamily testifies to the free communication between Europe, India and Central Asia during this period. In Europe it died out before the M. Pliocene, but persisted rather later in China, and in India until the U. Pliocene. In Central Africa *Giraffa* is found in the Pleistocene of Olduvai and is living to-day together with *Okapia*, a primitive member of the subfamily Palaeotraginae (Colbert, 1935*g*, p. 325).

A highly specialized branch of the Giraffidae, known as the Sivatheriinae, occurs in the L. Pliocene of Europe and India. These are characterized by their large size and complex horn development. I am inclined to think that this subfamily branched off from the Palaeotraginae, and that the earliest intimation of the horn development is found in the M. Miocene four-horned *Giraffokeryx* of India. Medium-sized representatives of it apparently occur in the U. Miocene of the same country, but it is not until the L. Pliocene that typical sivatherines, *Bramatherium* and *Hydaspitherium* of India and *Helladotherium* of Europe appear. They did not last in Europe beyond this, but in India *Sivatherium* is common in the U. Pliocene. No Indian occurrence later than that is known, but the genus appears to have survived in the Pleistocene of Central Africa (Hopwood, 1936, p. 641; Dietrich, 1937, p. 106). Large sivatherines appear to have existed in other parts of Africa. *Libytherium* Pomel (1893, p. 23), a rather progressive form, has been recorded from the Pliocene of Algeria and possibly Egypt (Stromer, 1905, p. 118), and *Griquatherium*, a specially large form from the Pleistocene gravels of the Vaal River in South Africa (Haughton, 1922, p. 15). Apparently at no time did this branch of the Giraffidae succeed in reaching Central Asia.

(3) *Protoceratidae*

This family, our information on which has been collected by Frick (1937, pp. 595–614), probably begins with *Heteromeryx* and *Pseudoprotoceras* in the lower beds of the White River (L. Oligocene) and ends with *Synthetoceras* in the topmost stage of the Miocene in the Clarendon of Texas. It has been classified by Schlosser in the Giraffoidea. This may be correct. It is very primitive in general structure, but its curious and often complex "horns" remind one of the Giraffidae, and may be an extremely precocious specialization allied to that group. They are confined to North America and need detain us no further.

XIII. THE CERVOIDEA

The earliest form possessing true cervid antlers, palmately branched on a long pedicle, is *Stephanocemas*, first described by Colbert (1936) from the M. (or U.) Miocene of Tung Gur in Mongolia. Stehlin (1937*b*, p. 193) generically identified with this a species from the M. Miocene of Stätzling, and fully confirmed Colbert's opinion that the antlers were deciduous. Later (Stehlin, 1939, p. 178) he referred to the same genus a smaller, more primitive species from the L. Miocene of the Sables d'Orléanais under the name of *S. infans*. *Dicroceros* and other allied genera, with antlers of a simpler, forked or dichotomously branched type, are not known before the M. Miocene both in Europe and Mongolia. The relation between such forms and the hornless genera which have been claimed as their ancestors has been discussed above (p. 155). Apparently there is an even greater hiatus here than in the Giraffoidea, but one must not overlook the possibility that some lagomerycid with simple, non-deciduous "horns" may have given rise to the periodically shed cervid antler. An initial stage of this process has been supposed by Lankester (1907) to exist in the "horn" of the okapi. *Dromomeryx* and *Aletomeryx* are too advanced on a giraffoid course to be the requisite cervid ancestor, but there is a strong presumption that Central Asia will one day supply the missing link.

Besides *Dicroceros*, recorded from Mongolia by Colbert (1936, p. 15), Cervidae belonging to many lineages abound throughout the Pliocene and Pleistocene of Central Asia (cf. Zdansky, 1925; Teilhard & Trassaert, 1937). Thence emigration into Europe, South East Asia and America has been continual. India does not seem to have received Cervidae to any great extent. They are unknown there during the Miocene, the horns referred by Pilgrim (1913, p. 313) to *Dicroceros* being those of *Giraffokeryx*. Cervulinae occur rarely in the L. Pliocene of India, their remains being as yet undescribed. Even in the U. Pliocene Cervidae are not common fossils, the ones so far described being ancestral to living species of the area. Cervidae seem to have penetrated to Africa in the L. Miocene, if the forms described by Stromer (1926, p. 115) and Arambourg (1933, p. 22) belong, as I surmise, to that family and not to the Bovidae. In Central Africa they did not survive into the Pleistocene, although in North Africa they still exist.

XIV. THE BOVOIDEA

In 1923 Schlosser divided the Bovidae, excluding *Merycodus*, *Antilocapra* and their allies, into two groups which he termed the Aegodontia and the Boodontia. Pilgrim (1939, p. 12) accepted this division, which is satisfactory not only for zoological reasons but also because it seems to coincide with a double developmental centre, Central Asia for the former and a region between India and Africa for the latter. Pilgrim (1939, p. 24) held that the available evidence was against the existence of the Boodontia previous to the M. Miocene, since the earliest bovid definitely known from Europe, India or Africa was *Eotragus* of the M. Miocene of Europe and of Lake Victoria (Pilgrim, 1939, pp. 25, 137). This is a primitive brachydont form, with short, upright horns, which might be ancestral to more than one subfamily, but seems to be nearer to the Boselaphinae. On the contrary, the high degree of differentiation shown in the L. Pliocene of Central Asia by the Aegodont subfamilies of the Ovibovinae and the Caprinae and in particular the occurrence in the probably M. Miocene beds of Tung Gur in Mongolia of a large and well-advanced species of caprine referred to *Oioceros* (Pilgrim, 1934) lead one to suppose that the Bovidae must have arisen in the L. Miocene.

I have suggested elsewhere (Pilgrim, 1941 *a*) the probability that the complex horn branching of the Merycodontidae of North America, in which the horn-sheaths were periodically shed, was preceded by a stage of simple, unbranched permanent horn-sheaths. Such a form as *Osbornoceras* with unbranched horns might conceivably never have shed its horn-sheath. This stage must have been essentially the same as that of the Caprinae. The hypsodont dentition and the structural resemblances between the M. Miocene Merycodontidae and a form like the Mongolian *Oioceros* (?) *grangeri* supports the view that they were actually derived from a primitive member of the Caprinae. The same may be said of the Antilocapridae, which did not, however, appear in North America until the L. Pliocene. They hardly overlap the merycodonts in time and the absence of probable ancestors in either case suggests that they are the result of two separate invasions, which, if we except North Eastern America, can hardly have come from elsewhere than Central Asia. It is significant that the Caprinae typically belong to that region and spread relatively late into other parts of the world: *Oioceros* into Europe and Persia in the L. Pliocene and *Nemorhaedus*, *Procamptoceras*, *Depéretia*, and *Megalovis* (Schaub, 1922, 1923, 1928) in the U. Pliocene; *Sivacapra* to India in the U. Pliocene and the nemorhaedines apparently even later; while they did not reach North America until the Pleistocene. There *Ovis* and *Oreamnus* still survive. In that case, however, the Merycodontidae and Antilocapridae must have died out in the country of their origin soon after they migrated to America, which is surprising.

The evolution of the Ovibovinae may with more certainty be placed in Central Asia. Numerous highly developed genera occur in the L. Pliocene of China, such as *Urmiatherium*, *Plesiaddax* and *Tsaidamotherium* (Bohlin, 1935 *c*, *d*). Allied forms penetrated into Europe contemporaneously as *Parurmiatherium* and *Criotherium*. None of these was of long duration, though the subfamily is recorded from the U. Pliocene of China, while *Ovibos* (musk ox) is by no means very rare in the European Pleistocene. The subfamily never reached India or south-eastern Asia, and North America only in the Pleistocene, where *Ovibos* and genera nearly allied to it are common in caves and interglacial deposits. The musk ox survives only in the extreme north of N. America and Greenland.

The Gazellinae were seemingly the earliest of the aegodont subfamilies to migrate from Central Asia. *Gazella* has been found in the M. Miocene of both India and Europe (Stehlin, 1937 *a*, p. 9; Pilgrim, 1939, p. 35). During the L. Pliocene numerous gazelline lineages existed in India, most of them very inadequately known. *Dorcadoxa*, possibly allied to the living African *Aepyceros* (impala), is one of the commonest. The most interesting of them is that of which the Indian *Antilope* (black buck) is the sole surviving representative (Pilgrim & Schaub, 1939).

Two genera are known from the L. Pliocene of India, one probably ancestral to *Antilope* and the other to the M. and U. Pliocene Chinese genera, *Antilospira* and *Spirocerus* (Teilhard *et al.* 1928, 1930, 1931, 1932, 1937). This group, which is characterized by twisted horns, is represented in the L. Pliocene of Europe and Persia by *Helicotragus* and *Protragelaphus*. The latter genus seems to belong to the same line as the widely spread European U. Pliocene genus *Gazellospira*, possibly also Chinese. *Spirocerus* persisted into the Pleistocene, having been found in the caves of Choukoutien. The distribution seems to indicate a dispersal centre situated between India and Africa.

The boodont Bovidae appear to have developed in the same region. The Boselaphinae and Tragelaphinae appeared simultaneously in the M. Miocene of India, both subfamilies being characterized by keeled and twisted horns. The Boselaphinae were differentiated in India into a variety of lineages, of which there is only evidence of two, those of the living *Boselaphus* (nilghai) and *Tetracerus*, the four-horned antelope, surviving into the Pleistocene. The most remarkable of these is that of which the M. Miocene *Sivaceros* is the earliest known member, and which became extinct with the ubiquitous L. Pliocene genus *Tragocerus*. We have some reason to believe that the horn-sheaths of these were sometimes forked, and therefore deciduous as in *Antilocapra* (Bohlin, 1935 *b*, pp. 8, 9). In the L. Pliocene of Europe Tragelaphinae were common but except for *Tragocerus* the Boselaphinae only occur sporadically; neither subfamily persists beyond the L. Pliocene. Except for *Tragocerus*, which probably arrived by way of Europe, and the U. Pliocene *Proboselaphus*, neither subfamily is known from Central Asia. *Duboisia*, allied to *Boselaphus* but of smaller size, was a Pleistocene immigrant to Java.

The Hippotraginae and Reduncinae, to which the sable antelopes and gemsboks and the water bucks and reed bucks of modern Africa respectively belong, are first known to occur in the U. Miocene of India. The former subfamily was common in the L. Pliocene of Europe, whence it probably migrated to Central Asia, where many lineages occur (Bohlin, 1935 *c*, pp. 107–56). They became extinct in Europe before the U. Pliocene, but both subfamilies abound in the U. Pliocene of India, though unknown in later deposits.

Alcelaphinae have not been found before the U. Pliocene—in India, the European L. Pliocene remains ascribed to the subfamily being very questionable.

Except the Boselaphinae all these boodont Bovidae have been found fossil in the Pleistocene of Central and North Africa (Pomel, 1893–4; Hopwood, 1934; Schwarz, 1937). They are little different from living species and are not very closely related to the U. Pliocene Indian forms. It seems that the Indian and African groups represent migrations from a common centre not very far to the west of India during the Pliocene.

The last subfamily of the Bovidae to be considered is the Bovinae (buffaloes and oxen). It seems almost certain that these originated in or near India in the U. Miocene from some member of the Boselaphinae (Pilgrim, 1939, pp. 136–7, 140–57). Their first actual occurrence in India and Europe is simultaneous, as *Proamphibos* (Pilgrim, 1939, p. 270) in India and *Parabos* (?) (Arambourg &

Piveteau, 1929, p. 117) in Europe, both being L. Pliocene. *Parabos* persists in the M. Pliocene of Europe and probably Egypt (Andrews, 1902, p. 437). These are primitive buffaloes, *Parabos* being probably a survivor of a somewhat less advanced type than *Proamphibos*. *Proleptobos*, a contemporary of *Proamphibos* and also Indian, is the earliest member of another bovine lineage. Its descendant *Leptobos* is the only type of ox met with in the U. Pliocene of Europe. The U. Pliocene fauna of India is, however, dominated by this subfamily. Not only does *Hemibos*, a direct descendant of *Proamphibos* occur, but also a primitive *Bubalus*, *Bucapra*, *Leptobos*, and *Platybos* and besides these the first true *Bos* and *Bison*, both in a more primitive stage than the species of *Bos* and *Bison* from the European Pleistocene. A recent find has disclosed the existence of *Hemibos* in Palestine, in a deposit of which the age is uncertain, but may be U. Pliocene (Pilgrim, 1941 *b*). Only *Bos* and *Bubalus* of a modern type occur in the M. Pleistocene beds of the Narbada in India. The dispersal of the bovines into other parts of the world began in the U. Pliocene. *Bison* and possibly *Bibos* occur in the U. Pliocene of Central Asia, and *Bubalus* is common in the Pleistocene. An advanced species of *Leptobos*, *Bibos* and *Bubalus* occur in the L. Pleistocene of Java. The Pleistocene of both North and South Africa have yielded remains of *Bubalus* allied to the Indian Pleistocene species, and Hopwood (1936, p. 639) has described a rather fragmentary skull, probably bubaline, under the name of *Bularchus* from the Pleistocene beds of Lake Victoria. The ancestry of *Syncerus*, the modern African buffalo, is still unknown. It belongs to a lineage quite distinct from the Indian buffalo, *Bubalus*, though both are probably descended from a primitive species of *Proamphibos*. The only bovines which emigrated to North America are *Bison*, which is represented by numerous species in the Pleistocene and is still living, and *Poephagus* (yak) (*fide* Frick, 1937, p. 568).

XV. SUMMARY

The modernized type of mammal, to which the Artiodactyla belong, invaded Europe and North America in the Palaeocene and L. Eocene. The practical identity of the L. Eocene faunas of these continents, to which the Dichobunidae are no exception, points to the invasion having come from a common source. The U. Palaeocene fauna of Mongolia is of a specialized ancient type, so that Central Asia could hardly have been that source. By a process of elimination Central Africa is indicated as the probable dispersal centre. Further proof of this is afforded by the immigrant character of all the Eocene and Oligocene Artiodactyla of Europe (excluding the Tragulina and the Pecora). These are probably descended from a primitive dichobunid. None of them is with certainty represented in Central Asia except the Anthracotheriidae and Suidae and that at a much later date. Only the Anthracotheriidae, Tayassuidae and Entelodontidae were received by North America equally with Europe. The endemic Artiodactyla of North America (Oreodontidae and Camelidae) were probably derived from primitive Dichobunidae. These were either confined to North America throughout their history or else were dispersed much later. The Anthracotheriidae were also derived from a primitive dichobunid in the L. Eocene and their radiative centre lay in south-eastern Asia, perhaps actually in India, whence their distribution was world-wide except into South America and Australia. Central Asia was the developmental centre of the Tragulina. Thence they spread over the whole of the Old World. The Pecora branched off from the Tragulina in the Oligocene. From their birth place in Central Asia most of the pecoran families sooner or later invaded other regions. The Giraffidae, however, never reached North America, while the Antilocapridae migrated only to that continent, and have remained there until to-day. Unlike the aegodont Bovidae which were essentially Central Asiatic, the radiative centre of the boodont Bovidae seems to have been in a region between India and Africa. Thence they eventually spread over the Old World. *Bison* and apparently *Poephagus* alone migrated to North America but not until the Pleistocene. The most specialized of the boodont Bovid subfamilies, the Bovinae (buffaloes and oxen), seems to have developed in or near India in the U. Miocene, whence their distribution was world-wide except to South America and Australia.

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FERTILITY IN MAMMALS AND BIRDS

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I. INTRODUCTION

THE rate of reproduction in a species or individual is regulated by a large number of factors which may take effect at different phases in the process of reproduction. In any analysis of fertility it is necessary first to ascertain the phase of the reproductive process at which a factor exerts its effect before any attempt can be made to control it. In this article it is proposed therefore to relate the various factors regulating fertility, so far as they are at present known, to three stages of the reproductive processes of mammals:

(1) Factors affecting the number of ova shed; these in the main are effected through the gonadotrophic hormones of the anterior pituitary.

(2) Factors affecting the number of ova fertilized; these in general not only include the number of sperm produced by the male, but also the time relations between mating and ovulation, that is, the conditions for the life of the sperm in the female tract.

(3) Factors affecting the number of embryos developing normally to birth; these include functions such as the progestin secretion from the corpus luteum, which is

necessary for attachment of the embryo and the maintenance of pregnancy, as well as genetic factors causing foetal atrophy.

II. NUMBER OF OVA SHED

It has long been known that the number of primordial ova present in the infantile ovary greatly exceeds those which ever have a chance of being fertilized during life. It has been found (Pearl, 1912) that the number of oocytes present in the ovary of the individual hen does not account for differences in the number of eggs produced. Kāppeli (1908), when seeking causes for the difference between the fertility of the wild boar and domestic pig, found rather that the difference lay in the number of large follicles which developed in the ovaries. Later several workers (Arai, 1920; Asdell, 1924; Slonaker, 1927; Lipschütz, 1927[1]) showed, by removing one, or one and a part of the other ovary, that the number of follicles which ripened at any one time did not depend on the mass of ovarian substance present. This was taken by Hammond & Marshall (1925) to indicate that the number of follicles which ripened depended rather on the level of a special substance circulating in the blood. As was shown later by Smith & Engle (1927), Zondek & Aschheim (1927) and others, this substance was the gonadotrophic hormone of the anterior pituitary gland. Polyovular follicles have been described in the ovaries of several species (Hartman, 1926; Engle, 1927), but there is no evidence that they play any part in affecting the fertility of the animal, rather their occurrence is symptomatic of a previously existing atrophy of the ovary causing the follicles to fuse (Loeb, 1917).

(1) *Gonadotrophic hormones*

Although a vast number of papers have been published on the gonadotrophic hormones of the anterior pituitary, they have been concerned for the most part with the separation of these hormones into follicle-stimulating and ovulating or lutealizing fractions and other aspects of purely theoretical interest. This also applies to the other gonadotrophic hormones such as the lutealizing hormone obtained from the urine of pregnant women and the follicle-stimulating hormone which is found in the blood serum of the mare between about the 45th and 100th day of pregnancy. It is only comparatively recently, however, that attempts have been made to use these hormones to increase the fertility of animals.

Knowing the effects of these hormones on the ovary it might seem to be a simple matter to increase the fertility of an individual by injection, but this is not the case. For example, Zawadowsky & Padootcheva (1935) found that they could cause superovulation by injections of 150–500 m.u. of pregnant women's urine hormone into ewes either 1–4 days before, during, or 2–3 days after oestrus, but in no case was more than one lamb produced. Lopyrin (1937, 1938), by injecting 100–400 m.u. of pregnant women's urine hormone into ewes 2–4 days before oestrus, was able to obtain superovulation, in most cases of 3–4 follicles, and in some cases up to 7–8 follicles. The lambing percentage was, however, only 20–60% higher than the controls and a high proportion of the ewes failed to conceive at all. As is pointed out below, failures in such cases may be due to lack of fertilization caused by faulty timing of ovulation in relation to mating (p. 12) or to lack of implantation due to lack of synchronization between the development of the blastocyst and the development of the corpus luteum (p. 15). Better results were obtained, however, by Loginova & Lopyrin (1938) by making two injections of pregnant mare serum hormone, the first on the 14th day after the previous oestrus (the ewe having a 16-day cycle), and the second at the onset of oestrus. Multiple ovulations were caused and fertility was considerably increased, quadruplets and quintuplets being born.

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[1] *Biological Reviews.*

The number of ovulations which can be obtained from a given dose of follicle-stimulating hormone is determined by conditions which are not yet fully understood. In the rabbit, a species which requires the stimulus of coitus acting on the anterior pituitary to set free the lutealizing hormone causing ovulation, multiple ovulations can easily be caused by three small daily doses of follicle-stimulating hormone followed on the fourth day by intravenous injection of lutealizing hormone (Pincus, 1939; Parkes, 1940). In the rat, a species in which the influence of the corpus luteum on the cycle is comparatively insignificant, it is only in immature animals that superovulation is caused by injection of gonadotrophic hormones (Cole, 1937); in these up to twenty-seven young were found in the uterus when the mothers were killed on the 17th–22nd days of pregnancy, while up to seventeen per litter were born alive.

In the sheep during the anoestrous period, when no corpus luteum exists in the ovary, injection of follicle-stimulating hormone (horse pituitary) causes the ripening of a large number of follicles, the exact number depending on the dose, but results in the ovulation of only the normal number for the species (Parkes *et al.* 1940). This is presumably because the lutealizing hormone necessary for ovulation, supplied by the sheep's own pituitary, is limited, or that the rupture of the ripest follicle or follicles inhibits the rupture of the others. From work in progress at the present time (Parkes *et al.* 1940) it would appear that this latter supposition may be correct, for the same amount of follicle-stimulating hormone gives very different results according to the time during the oestrous cycle at which it is injected. If injections are made 4 days after oestrus, when the corpus luteum is young and growing, no ovulation is obtained; if injection is made, however, at about the 13th day of the cycle, when the corpus luteum is beginning to wane, multiple ovulations of up to seven ova are obtained at the subsequent oestrus on the 16th day. It would appear that the existence of a waning corpus luteum is sufficient to prevent immediate ovulation of the first ripening follicle and gives time for many follicles to become mature before the trigger for ovulation is released. Up to seven fertilized eggs have been recovered from the tubes at 2 days, and five embryos from the uterus at the 3rd month have been recovered from ewes treated in this way (Parkes *et al.* 1940).

The discovery of anti-gonadotrophic hormones (Collip, 1938; Collip *et al.* 1940[1]; Parkes & Rowlands, 1936) in recent years opens up possibilities for limiting fertility, but the practical applications of these have not yet been fully explored.

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[1] *Biological Reviews.*

(2) *Age changes*

It is well known that only during the mid-period of the life cycle are ova ripened. In early life while growth is rapid the ovaries remain inactive, while in old age when senility sets in, the activity of the ovary dies down. Many investigations have been made to determine in different species the exact age of puberty, maximum fertility, and time of decline in ova production in old age, and a few examples of these may be quoted. In Merino ewes the percentage of twins rises from 4% at 3 years old to 9% at 5 years, remains at about the 10% level until the 10th year, and thereafter falls off rapidly to practically zero at the 12th year (Kelly, 1939). The age at which maximum fertility is reached has been found to be 150–180 days in rats (Feldman, 1926), 2nd litter in mice (Parkes, 1924), and to the 4th (Johansson, 1929), 5th (Yamane & Makita, 1934) or 6th (Zorn *et al.* 1933) litter in pigs, although Křiženecký (1936) has criticized the latter results in that they are affected by selection, and concludes that pigs in the maximum litter size is reached with the 2nd litter.

The causes for the lack of ova production in early life are not due to the age of the ovarian tissue itself, for Foa (1901) found by reciprocal transplantation of mature and young ovaries into young and mature rats that it was the age of the animal and not that of the ovary which determined the ripening of follicles. Later experiments by Zondek & Aschheim (1927), Smith & Engle (1927) and others, who transplanted or injected extracts of anterior pituitary glands, showed that immature animals could be made to shed their eggs by this means. It can therefore be concluded that the blood of immature animals is lacking in a sufficiency of the gonadotrophic substances of the anterior pituitary to cause ovulation.

Even after the onset of puberty is reached there is generally a period of time elapsing before the maximum number of ova are shed at ovulation. Hammond (1914) found that whereas young growing sows only averaged fourteen corpora lutea in the ovaries, adult fully grown sows averaged twenty. MacDowell & Lord (1925) also obtained similar results with mice, the rise in number of ovulations being especially rapid for the early litters. In general, the time of submaximal fertility corresponds to the time during which the skeleton is completing its full growth, and it therefore seems probable that some differential production between the growth hormone and gonadotrophic hormones of the anterior pituitary is involved; little exact information, however, is yet available on this point.

There is at present a division of opinion as to whether the prepubital anterior pituitary contains less gonadotrophic hormone than the adult gland; Lipschütz & Reynes (1932) found that there is less relative to weight in adult glands, while Wells (1938) finds that the potency of the prepubital glands is less than that of adults.

Within a species it is usual for the larger breeds to produce larger litters than the small breeds. Pickard (1930) found, for example, in rabbits that while the large Flemish averaged nine per litter and the moderate sized Chinchilla six, the small Polish averaged only just under four. This was also observed by Gregory (1932) and

Hammond (1934 *a, b*), the latter finding that a large strain of rabbits weighing 3500 g. shed on the average ten ova, whereas a small one averaging 1500 g. shed only five ova. This, however, does not apply between species; on the contrary, the larger the species, in general, the smaller the number of young that are produced at a birth.

Another activity of the anterior pituitary, that of the production of lactogenic hormone, seems also to be associated with inhibition of follicular ripening in some species such as the rabbit (Hammond & Marshall, 1925), rat (Kuramitsu & Loeb, 1921), and mouse (Kirkham, 1916–17), while it delays the onset of the breeding season in sheep (Roux, 1936). In other species, such as the cow, however, lactation appears to have no effect on follicular ripening.

(3) *The breeding season*

The rate of reproduction in many species is limited by the fact that breeding is confined to certain seasons of the year. In the domestic fowl, in which eggs are produced all the year round (instead of being limited to one season of the year as occurs in the wild jungle fowl), the rate of production is much greater at some seasons than at others. The main cause for the seasonal variation of egg production in the domestic fowl is probably the variation in hours of daylight at different times of year. Whetham (1933) has shown that the seasonal curve of egg production shows a close correspondence to that of the hours of daylight in different latitudes, while Kennard & Chamberlain (1931) and Hofman-Bang & Holm (1932) among others have shown experimentally that the winter egg production of fowls is increased by addition of electric light after dark. The ovaries of starlings are activated in winter by the addition of a light ration (Bissonnette, 1931), and this can be used to advance or prolong the egg production period in pheasants (Bissonnette, 1937).

The breeding seasons in mammals appear also to be controlled in the same way (Baker & Ranson, 1932). Just as plants can be classified into spring or autumn flowering according to whether the development of the flowering shoots are stimulated by lengthening or shortening daylight hours, so there exist in mammals species (ferret, horse) which only breed in the spring and summer and others which only breed in the autumn and winter (sheep, deer). When such species are transferred across the equator they either immediately, or after a short period for accommodation, fit in their breeding seasons to their new daylight environment (Marshall, 1936, 1937). As with birds, the addition of an extra light ration will cause summer-breeding mammals to commence breeding during the dark winter months (Bissonnette & Bailey, 1936, for ferrets; Bissonnette & Csech, 1937, for raccoons, etc.). It is evident that both the duration and intensity of the illumination are important (Marshall & Bowden, 1934), and that, provided these are constant, the particular part of the spectrum from which the rays come is immaterial, except that those coming from the ultra-violet end may have particularly lasting effects (Marshall & Bowden, 1936). Within the tropics, where the sun is overhead twice during the year, there are two periods of maximum ultra-violet light, and Baker

(1937) suggests that this may be the reason for the fact that many species in the tropics have two breeding seasons within the year. The mechanism of the action of light on the ripening of ova is thought to be by an effect on nerves in the region of the eye whereby the anterior pituitary is stimulated to increased activity. So far no experimental work has been published on the effects of shortening of daylight hours on the autumn-breeding animals in the way that Garner & Allard (1930) have done for the chrysanthemum, in which species there are critical lengths of daylight for different varieties. Kelly (1939), however, has shown that with sheep kept under the same light environment there are well-marked and constant differences between breeds and even strains in the time of onset and end of the breeding season.

Since the reason for the non-breeding season is fundamentally the lowered output of follicle-stimulating hormone by the anterior pituitary, the obvious method of extending fertility into this time of the year would appear to be by injections of follicle-stimulating hormone; but the matter is not so simple as it would appear at first sight. It is comparatively easy to cause the follicles to ripen and ovulate (Hill & Parkes, 1930), but other conditions are necessary, such as the occurrence of oestrus and the correct timing of this in relation to ovulation (see p. 176), before fertility can be induced; this applies particularly to the sheep, in which the first ovulation in the breeding season normally occurs without any external symptoms of oestrus (Grant, 1934). Zawodowsky *et al.* (1935 *b*) found that injections of gonadotrophic hormones into anoestrous ewes caused ovulation, usually between 24 and 36 hr. afterwards, but did not cause oestrus; they obtained some pregnancies by forced service or artificial insemination (see p. 174) on the first day after injection. McKenzie & Terrill (1937) state, however, that in such cases the time of ovulation may vary from under 36 to over 72 hr. after injection, and so it would appear to be necessary to inseminate such ewes on 2 or 3 successive days (see p. 176). Cole & Miller (1933) similarly found that ovulation in sheep could be induced during the non-breeding season by injection of 50 r.u. or more of follicle-stimulating hormone and that the ewes did not show any signs of oestrus. When a second injection followed the first after an interval of 17 days (the length of the oestrous cycle), however, both ovulation and oestrus were produced and a high percentage of fertile matings resulted. This result has been confirmed by Parkes & Hammond (1940), who also found that oestrus occasionally occurred after the first injection if there happened to be a corpus luteum in the ovary at the time of injection, cases of single ovulations unaccompanied by heat occasionally occurring in the non-breeding season.

(4) *Ovarian control*

The normal course of fertility is often interrupted by abnormal occurrences in the ovaries. In some cases the follicle does not rupture but goes on increasing in size and forms a cyst. Speaking generally, cystic ovaries are of two types, those with multiple small cysts and those with one or more large cysts; the former are usually associated with a nymphomaniac and the latter with an anaphrodisiac condition. In extreme cases the cow with cystic ovaries assumes male sexual characters (Calder, 1927). Not only do these cysts usually prevent further ovulation, but, in cases where they do not, the oestrin production by them may prevent implantation or cause abortion (see p. 179). In the cow these cysts may be ruptured per rectum or vaginam; if cysts are of long standing, however, they may re-form. Rupture of these newly formed cysts before the granulosa is lost leads, however, to the formation of a corpus luteum from them and so gives a period of rest during which the ovary can regain its normal condition (Marshall & Hammond, 1937). Cystic ovaries in mares can also be cured by this means (Day, 1939 *b*).

The cause of cyst formation has not yet been satisfactorily explained, but they have been experimentally produced in ewes and cows by the injection of large doses (2000 m.u. or more) of prolan (Zawadowsky *et al.* 1935 *a*, *b*): luteal cysts formed in this way may persist for some time in the ovaries of sows (Chivatov, 1935).

In most species the corpus luteum persists in the ovary throughout pregnancy and prevents the occurrence of oestrus during this time. In cows it is not uncommon for the corpus luteum to persist, in the absence of pregnancy, for 3–4 months and so cause a period of temporary barrenness; by squeezing these corpora lutea out of the ovary by the hand per rectum the inhibition is removed and the cows come into oestrus and breed in the course of 2–4 days (Frei & Stäheli, 1926; Hammond, 1929). The corpus luteum may be made to persist in the absence of pregnancy by the subcutaneous implantation of oestrin tablets (Hammond, Jnr., 1940). Even in cases where the corpus luteum does not persist, but the cow fails to become pregnant when served every 3 weeks, and where no visible sign of disease exists, the squeezing out of the corpus luteum about the 10th–15th day of the cycle cures the sterility in a large proportion of cases (Rumianzev, 1938; Hammond, 1939). The reason for this is not clear, but it seems possible that the removal of the corpus luteum throws the balance of ovarian secretions over to the oestrogenic side and so intensifies the oestrous changes in the uterus and cervix (see p. 178).

III. NUMBER OF OVA FERTILIZED

Various factors affect the number of ova fertilized. Not only is the number and vitality of the sperm produced by the male important, but all those factors which make for easy ascent and prolongation of the life of the sperm in the female tract come into play. Not the least of these is the time relations that exist between mating and ovulation, for both the sperm and ovum have a very limited independent life and, unless time relations are properly synchronized, the chances of fertility are considerably reduced.

Because only one sperm is required to fertilize an ovum, and because normally very large numbers of spermatozoa exist in the ejaculate, it was formerly considered that the male was relatively unimportant as a determiner of fertility, and that with the male it was a question of either full fertility or sterility. Recent work, however, has shown that this is not the case and that the production of large numbers of vigorous sperm is essential for maximum fertility. Walton (1927), for example, by successively diluting semen and inseminating doe rabbits, found that the size of the litter varied directly with the density of the sperm suspension, and that with a higher degree of dilution complete sterility resulted, even though the suspension contained many hundred thousands of spermatozoa.

(1) *Sperm production*

Much sterility and lowered fertility is caused by defective sperm production in man (Moench & Holt, 1931), stallions (Walton & Fair, 1928), bulls (Lagerlöf, 1934), rams (McKenzie & Phillips, 1934; Constock & Brady, 1937), boars (McKenzie *et al.* 1938) and other species. Nicol (1934), for example, finds in the guinea-pig that the "prenatal mortality" is 29 %, which is chiefly due to lack of fertilization of the ova shed. In such cases, the sperm may be deficient in number, abnormal in their form, reduced in vitality, or a combination of these conditions may exist. Methods for detecting these are described by the workers quoted above. The technique employed consists, after collection by the artificial vagina method (see p. 174), of volume determinations, sperm counts to determine density, staining methods to detect abnormalities and determination of the rate of gaseous exchange under standard conditions (Walton & Edwards, 1938) to determine vitality. When the sperms are inactive, and the alkaline secretions of the accessory glands predominate in the ejaculate, the pH of the semen becomes more alkaline (7·0 and over): this can be used as a field test for fertility in rams (Webster, 1938, 1939). For quick estimation of the numbers of sperm in a sample of semen a sediment method has been used by Gunn (1936) and an opacity method described by Burbank (1935).

Various causes for defective sperm production have been suggested. Cases have been reported where the sperm deficiency in the semen is due to infections in or blocking of the tubules of the epididymis (Savage, 1929; Anderson, 1938). Dietary deficiencies of one or other vitamins, and especially vitamin E (Kennedy, 1926 *a*; Korenchevsky, 1933; Mason, 1939), cause lack of sperm production under experimental conditions; but such cannot be the cause of those cases which are frequently found on perfectly normal diets. Phillips & Andrews (1936) found that poor nutrition retards the production of spermatozoa in the boar, while Cunningham & Hopkirk (1935) obtained sterility in male rats by feeding excessively high-protein diets.

Most of the Russian workers, however, agree that the feeding of animal proteins (milk, blood, eggs, etc.), as distinct from vegetable proteins, increases the sperm production of bulls, rams and boars (Smirnov-Ougryumov, 1937; Lissov *et al.* 1937; Hudiakov, 1938).

While removal of the anterior pituitary causes cessation of sperm formation (Hill & Parkes, 1932; Allanson *et al.* 1935; Liu & Noble, 1939), the results of injections of gonadotrophic hormones on sperm production are conflicting, and in practice, so far as increasing fertility goes, disappointing. For instance, Moore (1936) obtained no results from injections on sperm production in the rat, while Witschi & Keck (1935) obtained sperm formation in the testes of the sparrow in the non-breeding season by injection of follicle-stimulating hormone, but none from injections of lutealizing hormone. Evidence that special substances circulating in the blood stream form the basis of the level of sperm production by the testes too is still far from complete. While removal of one testis leads to compensating hypertrophy in the remaining testis in cocks and drakes (Domm & Juhn, 1927; Benoit, 1931) the evidence is not clear in mammals. Smelser (1933) found increased sperm production from the remaining testis in rats, but his results have been criticized by Edwards (1940), who on careful quantitative investigation found no effect at all on the sperm production of the remaining testis in rabbits when one was removed.

The rate of sperm production by the testes does, however, vary greatly at different seasons in the year in most birds and in some mammals; these changes can be induced by changes in the light ration, in the same way that the production of ova by the ovary can be influenced (Bissonnette, 1931, starling; 1935, ferret; Riley, 1937, sparrow). By injections of pregnant women's urine hormone following light treatment, fertility and pregnancy have been produced during the non-breeding winter months in ferrets (Allanson *et al.* 1934).

Small differences in temperature have a great effect on the production and life of the spermatozoa in mammals. Crew (1922) was the first to suggest that the higher temperature that exists in the abdomen as compared with the scrotum was the cause of the lack of sperm production by abdominal testes. Experimentally, the placing of the testes in the abdomen causes atrophy of the tubules (Moore, 1926 *a*; Cunningham, 1927); this degeneration is produced in 10 days (Leonard & Hamilton, 1937), but on replacement in the scrotum is not regained until after 90 days (Moore, 1926 *b*). Warm water (46° C.) run over the scrotum is sufficient to cause degeneration of the sperm (Young, 1927). After raising the temperature of the scrotum of the ram, Phillips & McKenzie (1934) found that it took from 4 to 13 days for abnormal sperms to appear in the ejaculate; they suggest that a heavy fleece or high "condition", by raising the temperature of the testes, frequently causes lowered fertility in rams.

As the sperms pass from the testis through the long tube of the epididymis to the lower part of the vas deferens they undergo a process of maturation and, if not ejaculated, of dissolution. Sperm taken from the testes are not usually capable of fertilizing ova (Young, 1931; Munro, 1938 *b*); Young (1931) demonstrated the necessity for the maturation of sperm in the epididymis. Guinea-pigs inseminated with immature sperm from the testicular end of the epididymis give only 33% fertility as compared with 68% with mature sperm from the proximal end; when, however, the sperms are confined in the epididymis for longer periods by ligation, conditions are reversed and the overripe sperm from the distal end only give a 25% fertility. Sperm maintain their motility in the lower epididymis up to 60 days and their power of fertilizing ova up to 38 days in the rabbit (Hammond & Asdell, 1926), and 42 and 21 days respectively in the rat (White, 1933); these times for the guinea-pig (Benoit, 1925; Moore, 1928; Young, 1929) and bull (Kirillov & Morosov, 1936) are very similar. These facts have a bearing on the problem of what is the optimum period between ejaculates for obtaining sperm of maximum vigour: Kirillov & Morosov (1937), for example, find that in bulls the second ejaculation, as regards all sperm indices, is better than the first one which may be very variable.

McKenzie *et al.* (1938) concluded that in normal boars the maximum production of effective sperms occurred when the time between copulations was at intervals of 48 hr. or more, but there was an indication that in boars of low spermatogenic activity the optimum interval was somewhat longer. The number of sperms too in the ejaculate falls rapidly in frequent collections at short intervals: in rabbits Macirone & Walton (1938) found that the number of sperms in the ejaculate fell from 73 to 13 millions from the 1st to 6th ejaculation made within a day. Such "exhaustion tests" form the best means of measuring male fertility (Walton & Edwards, 1938); the number and vitality of the sperms in the epididymis cannot be properly tested from one collection alone, but the exhaustion test soon shows up differences between males in this respect (McKenzie & Berliner, 1937, for rams; Belding, 1934, for man).

(2) *Artificial insemination*

The fertility of males of high genetic worth can be enormously increased by the new methods of artificial insemination. For example, it has been found possible on the average to inseminate successfully about two sows, eight mares, ten cows and thirty-five ewes from one ejaculation of their respective males (Milovanov, 1934, 1938 *a*). This aspect of artificial insemination is of far more importance than its previous main objective, which was the cure of certain forms of sterility (Siebenga, 1938). Cases have been reported where up to 2600 ewes have been successfully impregnated from one ram in one season (Neumann, 1935).

Various methods for the collection of the semen have been employed, the main object being to collect the ejaculate *in toto* and free from contamination. Many forms of artificial vagina have been invented (Kusnetzowa *et al.* 1932; Milovanov, 1934; Neumann, 1935; Sørensen, 1938; Walton, 1938 *a*) for the male to serve into as he jumps the female. In place of a female in oestrus a stuffed dummy may be substituted if a conditioned reflex has been established in the male (Walton, 1933; Olbrycht, 1935; Bonadonna, 1937), or a female not in oestrus can be used if she is confined in a crate so as to prevent movement (Edwards & Walton, 1938). In bulls semen can be obtained by massage of the vasa differentia with the hand per rectum (Miller & Evans, 1934), and in rams by electrical stimulation of the cord at the level of the 4th lumbar vertebrae (Gunn, 1936). Methods for collecting from birds have been described, among others, by Nikitin (1932), Warren & Scott (1935), Grěcka (1935), Burrows & Quinn (1935, 1937) and Griffini (1938). As the semen is injected directly into the cervix in artificial insemination, much less is necessary than in a normal mating where, in most species, it is sprayed over the surface of the vagina and only a little of the ejaculate finds its way into the uterus. Where a large number of females are to be inseminated from one ejaculate the semen is diluted with isotonic salt solutions and glucose, the exact composition varying with the species (Milovanov, 1934). Experience has indicated, however, that over-dilution is harmful to fertility, and so the recent tendency has been to inseminate with very small amounts of undiluted semen; for this purpose a micro-inseminator has been invented (Milovanov, 1938 *a*). Another method recently adopted has been to mix the sperm with gelatine at body temperature and allow it to solidify in small tubes; on insemination the pessary is put in or through the cervix, the sperm being liberated when the gelatine melts (Milovanov, 1938 *b*).

Many experiments are being made on methods to preserve the fertilizing power of the sperm outside the body. As mentioned above, the power of fertilizing ova disappears in the sperm long before their motility. With the development of air transport long distances can be covered in a short time, and as the cost of transporting sperm is far less than that of transporting the larger domestic animals, there has been an urge to develop methods. The object of most of these methods is to conserve the energy contained within the sperm itself by reducing its motility during the time that it is being kept outside the body. In the epididymis, where the sperms are immotile, the fertilizing capacity may be maintained up to 40 days (p. 173) but nothing approaching this time has yet been achieved *in vitro*; motility, as distinct from fertility, has, however, been maintained for 64 days in ram's sperm kept outside the body (Gunn, 1936). The methods used, like those adopted by the hibernating animal, consist in raising the carbon dioxide tension to that of tissue as distinct from pulmonary level and in lowering the temperature; the sperm is collected under liquid paraffin and kept at a temperature of about 10° C. (Hammond, 1930; Walton, 1930). For the sake of convenience, in many experiments the temperature has been maintained at 1–2° C., but recent work (Komarov & Gladtzinova, 1937; Milovanov *et al.* 1939) has tended to show that the higher level is best. More recently it has been found that rapid cooling causes temperature shock to the sperm (Birillo & Pukhaisky, 1936), and that sperm will keep well at 1–2° C. provided it is cooled slowly and given time to acclimatize (Chang & Walton, 1940).

The length of time that the fertility of the semen is able to be maintained outside the body varies in different species according to the proportion of the sperm to accessory gland fluid in the semen (Hammond, 1936 *b*), for the accessory gland fluids activate the sperm. Attempts have therefore been made to prolong the life of the sperm by eliminating these secretions: this can be done either by collecting the semen in fractions as it is ejaculated (Gunn, 1936; Miller & Evans, 1934; McKenzie *et al.* 1938) or by centrifuging the sperm away from the accessory fluids after collecting (Walton, 1938 *b*). Lambs have been obtained from sperm stored for 6 days in the laboratory (Winters *et al.* 1938) and up to 8 days from ram sperm sent from Washington, D.C., to Idaho (Phillips *et al.* 1938), while young have been produced from ram sperm sent from England to Poland (Prawochénski & Walton, 1935) and from bull sperm exchanged between England and Holland (Edwards *et al.* 1938).

(3) *Time relations between mating and ovulation*

In most cases the time of mating is strictly limited to the short period of oestrus which occurs just before ovulation. By experimentally altering the normal time relations between insemination and ovulation it has been determined that in most mammals the separate life of sperm and ova in the female tract is of very limited duration. These facts form the explanation of many cases of low fertility and sterility.

In the domestic fowl, in which the egg takes about 24 hr. to pass down the reproductive tract, no egg laid within 21 hr. after the time of mating is fertile (Dunn, 1927; Chlebaroff, 1930), that is, the egg must be fertilized soon after it is shed if it is to be fertilized at all. Iwanow (1924) went so far as to say that the growing oocytes in the hen were fertilized in the ovary. Walton & Whetham (1933) could not support his conclusion, while Hartman (1937) has pointed out that experiments by Warren & Kilpatrick (1929) definitely show that this is not so, for when the cock is changed, eggs fertilized by the second cock were laid as early as the second day after the change had been made.

In the rabbit the ova are shed 10 hr. after mating; if the first mating to induce ovulation is made with a sterile male and a second at a certain interval of time afterwards with a fertile male, the exact time that the ovum remains capable of fertilization can be determined, for in the rabbit the spermatozoa take some 4 hr. to reach the tubes. The ovum in the rabbit remains capable of fertilization for 6 hr. only after ovulation, for matings made later than 2 hr. after ovulation fail to produce young (Hammond, 1934 *b*); it is suggestive that it is for about this length of time that the ova remain in the ovulation plug at the top of the Fallopian tube (Pincus, 1936). Small litters are produced when the mating is made between 3 hr. before and at the time of ovulation. In the ferret the ova remain capable of fertilization for a longer period—some 30 hr. (Hammond & Walton, 1934), while in the guinea-pig the limit of ovum viability after ovulation is 26 hr. (Young & Blandau, 1936). Lowered fertility also follows matings after the 30th hour subsequent to the onset of oestrus in sheep (Quinlan *et al.* 1932 *a*), ovulation occurring in the sheep at from 24–36 hr. after the onset of oestrus (McKenzie *et al.* 1933). In the mare, in which ovulation occurs about 24 hr., or slightly more, before the end of oestrus (Mirskaya & Salzman, 1935), the percentage of fertility is very low (20%) for matings made during the last 24 hr. of oestrus (Hammond, 1938 *b*). Day (1940) could not obtain fertility when mares were inseminated 2–4 hr. after ovulation as determined by rectal palpation of the ovaries, although high fertility was obtained from inseminations made 12 hr. before ovulation.

Ovulation in the monkey occurs about the 14th day of the cycle, and Hartman (1932 *b*) finds that in this species the bulk of conceptions occur between the 12th and 15th days of the cycle. In woman ovulation occurs between the 12th and 14th days of the cycle (Allen *et al.* 1930). Knaus (1934), on the basis of numerous records, concludes that the period during which conception can take place in woman consists of the ovulation period (12th day for a 26-day cycle to 16th day for a 30-day cycle) plus 3 days before it and 1 day after it: the rest of the cycle he considers a "safe period".

The length of time that the sperm remain capable of fertilizing ova in the female tract varies greatly in different types of animals. In general these differences appear to be related to temperature conditions. In fish, which of course are poikilothermal, van Oordt (1928) found that fertilized females of *Xiphophorus helleri*, kept isolated from males for more than 10 months, gave birth to several subsequent litters. In hibernating mammals such as bats, where the body temperature falls during winter, the spermatozoa are said to live through the winter in the uterus and fertilize ova in the spring (Courrier, 1927; Redenz, 1929). In birds, where the testes are abdominal and so normally subjected to high temperatures, the life of the sperm in the female tract is also comparatively long. The turkey lays a clutch of 20–30 eggs in about as many days, and these are all fertilized as a result of one treading. In the domestic fowl fertile eggs may be laid up to 10–20 days after a mating, depending on the number and vitality of the sperms of the cock, although the percentage fertility begins to fall off rapidly after about the 5th–7th day (Crew, 1926; Chlebaroff, 1930). In ring-doves the sperm retain their fertilizing power for 8 days (Riddle & Behre, 1921), and in ducks for 7–11 days (Chappellier, 1914).

In mammals, however, with scrotal testes, where the temperature is normally below that of the abdomen, the sperm have a very much shorter life in the female tract. The sperm remain fertile in the rabbit for only 30 hr. (Hammond & Asdell, 1926), while after 24 hr. in the female tract small litters are frequently produced as a result of the number of viable sperms not being sufficient to fertilize all the ova shed. Feebly motile sperm have been found in the uterus and tubes of the guinea-pig up to 41 hr. after copulation and up to 17 hr. in the rat (Yochem, 1929), while slightly motile ones have been found in the bitch up to 2½ days (Whitney, 1927). Kelly (1937) finds that the fertilizing power of sperms, even from the most fertile rams, is lost at about 34 hr. after copulation, while Lopyrin (1938) gives this time as 36–40 hr. Quinlan *et al.* (1932 *a*, *b*) believe that in the ewe the cervix acts as a reservoir for sperm from which there is a continuous issue up the tract: they too find that by 48 hr. after mating all the sperm in the female tract are immotile.

In the horse, in which the life of the sperm in the female tract may be rather longer, up to at the most 5 days, the scrotum is held closely to the abdominal wall (Hammond, 1938 *a*). In the mare oestrus may be of long duration, but it has been found that fertility decreases from about 67 % when mating is made 1 day before ovulation, to 50 % at 4 days, and 29 % at 5 days before ovulation, while all matings made 7 days or more before ovulation proved sterile (Hammond, 1938 *a*). This is probably the reason why the travelling stallion, which is mated with mares at any time during the period of oestrus, has a lower percentage fertility (53 %) than the thoroughbred (66 %) which stands at stud and tries mares every 2 days, and still lower than the mountain pony (95 %) which runs freely with the mares all the time. Constantinescu & Mauch (1936) found that mares mated once only during an oestrus gave only a 40 % fertility, but when mating was made twice, on the 2nd and 5th days of oestrus, the fertility was raised to 57 %. By inducing ovulation at a known time in relation to the time of mating by injections of lutealizing hormone (1000 m.u.) the chances of obtaining fertility in horses is increased (Mirskaya & Kedrov, 1938; Day, 1939 *b*).

In monkeys the chances of fertility from a mating rise from a low level just before or after menstruation to a peak about the 12th day after the beginning of the menstrual flow, ovulation occurring about the 13th–14th day (Hartman, 1932 *b*). In woman, too, much the same condition exists (Ogino, 1932; Knaus, 1934).

(4) *Other factors*

It will be realized from what has been said above that the chances of fertility from a mating made at a certain time before ovulation are greatly affected by the quantity and quality of the sperm introduced; hence the attention which is now being given to fertility in the male (p. 172). Conditions in the female tract, such as the presence of inflammations and leucocytes or the incomplete liquefaction of the mucus of the cervix at oestrus (Hammond, 1939), which make it difficult for the sperm to ascend the tract, also cause lowered fertility for the same reason. While Green & Winters (1935) find that sperm reach the infundibulum of the non-oestrous ewe almost as quickly as in the oestrous ewe, Lopyrin (1938) concluded that they could not pass up the tract in the non-oestrous ewe. Sjövall (1938) finds too that in guinea-pigs the passage of the sperm through the cervix is practically excluded in the non-oestrous phase. It has been found that in mares which suck air into the vagina, the tract is liable to become infected and sterility results. If the upper commissure of the vulva is sutured so that the normal semi-anaerobic state of the reproductive tract is regained, the inflammatory condition of the tract clears up and the mare becomes fertile again (Caslick, 1937; Day, 1939 *b*).

A vague condition known as incompatibility was often cited by older writers as a cause of sterility. While much of this may have been due to lack of sperm or other conditions mentioned above, it has been suggested by Guyer (1921), McCartney (1923), Walsh (1925) and Pommerenke (1928) that spermatoxins produced in the female are concerned. No very convincing evidence exists, however, that these conditions operate to produce sterility under natural conditions.

IV. NUMBER OF EMBRYOS DEVELOPING TO BIRTH

During the course of pregnancy in mammals and incubation in birds, many factors may come into play to prevent the development of the embryo. In the mammal, defects in the mechanism of attachment, and later in the supply of those hormones necessary for the maintenance of pregnancy, may lead to absorption or abortion of the young, while in both mammals and birds lethal genetic factors may cause death of the embryo before birth or hatching. In certain special cases the number of young produced at birth is greater than the number of eggs fertilized. In the armadillo (Stockard, 1921; Hamlett, 1933), divisions in the egg after fertilization lead to the production of four embryos. In other species this phenomenon is the exception rather than the rule, although cases of identical twins, that is, twins derived from a single fertilized ovum, are by no means uncommon in certain species of mammals. The evidence for this is based for the most part on the mirror images of physical characters in the twins. Such cases have been described in man by Newman (1923), Newman *et al.* (1937), and in cattle by Gowen (1922), Lush (1929), Hutt (1930) and Kronacher (1936). In the marmoset monkey, of whose reproduction twinning is a special feature, Wislocki (1932) inclines to the view that the twins are as a rule derived from a single ovulation. In this connexion it is perhaps significant to note that those who have investigated the genetic history of multiple births in man (Davenport, 1920; Greulich, 1934, 1938) find that the records of twinning are quite as marked, or even more so, on the father's side of the pedigree than on the mother's.

(1) *Failure of attachment*

In most species of mammals the fertilized eggs take about 3 days to travel down the tubes and pass into the uterus. If oestrin is injected into mice and rabbits during this period, however, the blastocysts become "tube-locked" and perish (Burdick & Pincus, 1935; Pincus & Kirsch, 1936; Whitney & Burdick, 1936; Burdick *et al.* 1937). The tubal journey of ova is shortened, on the other hand, by about 12 hr. in pseudo-pregnant rabbits in which a second ovulation has been induced by gonadotrophic hormone (Wislocki & Snyder, 1933).

In order that implantation of the blastocyst shall occur, the uterine mucosa has to be in a receptive state and this is brought about by the progestin secreted by the corpus luteum. Under certain conditions this secretion is absent or deficient in amount and so leads to failure of the fertilized ova to become implanted. The condition may be temporary only, as occurs in the rat and mouse during a certain period of lactation (King, 1913; Kirkham, 1916–17), merely causing a prolonged pregnancy, or may be more lasting (as in the rabbit, Hammond & Marshall, 1925) and cause lack of implantation or atrophy and absorption of the developing embryos. Brambell (1937) is of the opinion that this failure of the blastocysts to develop in some species during lactation may be due to lack of a growth-stimulating substance in the uterine lumen. In the sheep (Litovchenko, 1936), cow and many other species, however, coincident lactation does not interfere with normal development of the young.

The failure to obtain pregnancy after injections of gonadotrophic hormones during the cycle in cows and in sheep (Zawadowsky *et al.* 1935 *a*, *b*) may be due to the fact that the uterine mucosa is not in a receptive stage when the ova are ready to be implanted. The corpus luteum already in existence in the ovary takes priority over the newly formed corpus luteum induced by injections of gonadotrophic hormones and the normal rhythm of the cycle is not interfered with by these injections (Zawadowsky & Eskin, 1937). When in the cow the corpus luteum in existence is removed during the cycle, ovulation occurs in the course of a few days and pregnancy ensues from matings, for the normal rhythm of the cycle is changed. It is probable that the uterine changes concerned with implantation are rightly timed when the corpus luteum is removed, but are not so when a cyclic corpus luteum is already in existence at the time of the artificial ovulation.

The exact duration of time over which implantation is possible after ovulation in different species still needs working out. In rabbits injected with prolan on the 7th day of pseudo-pregnancy and inseminated, Wunder & Padootcheva (1936) failed to obtain pregnancy, although, without considering the implantation aspect, they attributed the failure to prevention of the sperm passage up the tract (see p. 178). By injecting folliculine-benzoate together with the pregnant mare serum into cows during the cycle Zawadowsky & Eskin (1937) found that the uterine mucosa was prepared and the obstacle for conception was removed; these conclusions, however, are based on the results of a few cases only and need confirmation.

(2) *Absorption or abortion*

Injections of oestrin given during early pregnancy will override the action of progestin from the corpus luteum and so lead to a failure of the ovum to form an attachment to the uterus; if given later in pregnancy oestrin will cause absorption or abortion in rats, mice, rabbits and cats (Smith, 1926; Parkes & Bellerby, 1926; Courrier & Kehl, 1933; Courrier & Raynaud, 1934; Courrier & Gros, 1935 *b*; Robson, 1935 *a*, *b*). Reynolds (1937) finds that the disproportions between the growth increments of the foetus and uterus during the last part of pregnancy are due largely to the increase in the influence of oestrin with the advance of pregnancy; as these disproportions are concerned with the onset of labour, abnormal rises in the level of oestrin in the blood may account for cases of spontaneous abortion.

Removal of the ovaries or destruction of the corpora lutea during the early stages of pregnancy also leads to atrophy and absorption of all the embryos (Fraenkel, 1903; Weymeersch, 1911; Hammond & Marshall, 1925; Courrier & Gros, 1935 *a*). The effect of removal of the ovaries during the later stages of pregnancy after the placenta has been fully formed varies, however, in different species. In those in which the corpus luteum persists throughout the whole of pregnancy, for example in the goat (Drummond-Robinson & Asdell, 1926), cow (Hess, 1921; Schmaltz, 1921), bitch and rat (Marshall & Jolly, 1906), spermophile (Drips, 1919), rabbit (Hammond & Marshall, 1925) and mouse (Parkes, 1928; Robson, 1936), abortion invariably results. In woman, in which the corpus luteum disappears during the middle of pregnancy, removal of the ovaries during the latter part of pregnancy, however, does not necessarily cause abortion (Bell, 1920; Ask-Upmark, 1926); it will probably be found that the mare will act in the same way, for in this species also the corpus luteum disappears in mid-pregnancy. The guinea-pig (Herrick, 1928) is also known to behave in this way; in the cat, too, removal of the corpora lutea or ovaries after the 49th day does not interrupt pregnancy (Courrier & Gros, 1935 *a*).

It is believed by some (Selye *et al.* 1935; Haterius, 1936) that progestin is secreted by the placentae as well as by the corpus luteum, for they find that, in animals such as the rat, pregnancy may be maintained following removal of the ovaries shortly after mid-pregnancy if all but one of the foetuses are removed, provided the additional placentae (of the removed foetuses) are allowed to remain *in situ*. Possibly related to this is the fact that in the horse, a species in which only about fifteen twin births per thousand occur (Lauprecht, 1932), more than one-half of the twin pregnancies end in an abortion (Wriedt, 1928). Absorption of the embryos is also caused by lack of vitamin E in the diet (Evans & Bishop, 1923; Mason, 1939).

During the second half of gestation the anterior pituitary appears to have no action in maintaining pregnancy. Hypophysectomy made during the second half of pregnancy in the rat (Pencharz & Long, 1933), mouse (Selye *et al.* 1933), cat (Allen & Wiles, 1932), guinea-pig (Pencharz & Lyons, 1934), and ferret (McPhail,

1935) has shown that pregnancy can continue uninterrupted for its normal duration in the absence of the anterior pituitary. Walton & Hammond (1934) found too that in ferrets pregnancy could be maintained in the non-breeding season at a time when the gonadotrophic activity of the anterior pituitary was absent. In the early stages of gestation, however, hypophysectomy quickly leads to the termination of the pregnancy, probably through its action on the activity of the corpus luteum (see p. 179).

Pathological conditions of the uterus and cervix have been cited as the cause of much sterility in cows (Quinlan, 1929), but pathological organisms are not a constant feature of such cases and there has been as yet little investigation as to the causes of the metritis in the uterus or how it prevents implantation of the blastocyst. Preliminary work on rabbits, however, suggests that these symptoms of metritis and cervicitis can be caused by subcutaneous implantations of oestrin tablets (Hammond, Jnr., 1940).

While in many cases the cause of an abortion may be well known, the mechanism by which the result is achieved is frequently but little understood. For example, it is well known that contagious abortion in cows may be due to Bang's bacillus (Nielsen, 1926) or *Trichomonas* (Riedmüller, 1933), but it is not known why the abortion occurs. It is possible that the result depends on the destruction of the placentae affecting the production of the hormones necessary for the continuation of pregnancy, but this is still a relatively unexplored field (Vogt-Möller, 1939).

(3) *Foetal atrophy*

Lethal genetic factors in many species may cause the death of the embryos at or before birth or hatching: in such cases some only of the embryos perish, but not all, as under the conditions mentioned above. The finding of unexpected Mendelian ratios when yellow mice were bred together led Kirkham (1919) and others to the belief that the homozygous condition for yellow was lethal. Such lethal Mendelian characters which cause uterine death of the embryos have been described in many species (mouse, Little, 1920 and Dunn, 1937; cat, Jones, 1922; horses, dogs, sheep and cattle, Mohr, 1929 and Wriedt, 1930); they have also been found in birds (fowl, Dunn, 1923; Hutt & Greenwood, 1929). They are almost all recessive in inheritance, and it is for this reason that fertility is frequently reduced by inbreeding. For example, on inbreeding guinea-pigs for 25 years, Eaton (1932) found a decrease of fertility which was associated with an increase of prenatal mortality. In those species which produce only one young at birth, absorption or abortion of the embryo results from its death, but in those species in which many young are produced the dead and mummified embryos are carried in the uterus to full term and may be found mixed with the cleansings after birth (Hammond, 1914, 1921).

In the rabbit, foetal atrophy is inherited as a maternal character and not as a homozygous embryonic condition as in the cases mentioned above. This is shown by the fact that does carrying this character produce the same number of atrophic young whether they are mated with bucks of their own or unrelated strains (Hammond, 1928). The embryos die off at different stages during the course of development, and there is some reason to suppose that their death may be caused by the deficiency of some substance in the mother's blood; for if one tube is cut so as to reduce the number of ova fertilized, a much smaller percentage of foetal degeneration occurs than in normal control animals where a larger number of ova are fertilized. Some support for this suggestion is to be found in experiments made in conjunction with Prof. G. Pincus. By injecting with follicle-stimulating hormone, followed by an injection of lutealizing hormone at the time of mating, the fertilization of 30–40 ova can be obtained in strains of rabbits which normally produce ten. In such cases, however, foetal atrophy occurs at various stages of development, which reduces the number of embryos to about the same as that which the doe would normally produce. Such results seem to point to the conclusion that the number of young that develop normally to birth depends on the amount of some substance in the blood which acts as a limiting factor; but what this substance may be is not yet known. Among women it is well known that some individuals show a tendency to repeated abortions (Vignes, 1923), and it may be that some deficiency of the same nature is involved.

Long ago it was suggested (Stephenson, 1885) that foetal death might be caused by lack of vigour in the sperm, which reduced vitality in the eggs fertilized by them, and others have since taken this view. No evidence, however, has been found either in ring-doves (Riddle & Behre, 1921), rabbits (Hammond & Asdell, 1926) or fowls (Dunn, 1927), indicating that weakness or modified viability in the embryos is obtained from fertilization with stale sperm. Young & Blandau (1936), however, find in guinea-pigs that the age of the ovum at fertilization affects the viability of the embryo: this is first expressed when the ovum is 8 hr. old, and although the ovum may be fertilized up to 26 hr. after ovulation, normal young are not produced from ova more than 20 hr. old. They find that as the ova become older before fertilization, so the pregnancies tend to be terminated at an earlier stage, and suggest that this may be one of the causes of abortion in women (Blandau & Young, 1939).

V. SUMMARY

1. The numerous factors which influence fertility in animals are best considered in relation to the stage of the reproductive process at which they take effect.

2. The various factors which affect the number of eggs shed do so mainly by means of changes in the level of follicle-stimulating hormone circulating in the blood. The breeding season of certain species is probably controlled mainly by variations in the duration of daylight, which stimulates through nerves the output of gonadotrophic hormones from the anterior pituitary.

3. The number of ova fertilized depends not only on the number and vitality of the sperm produced by the male, but also on the time relations existing between insemination and ovulation, since both the sperm and ovum have a very limited independent life. The fertility of males can be enormously increased by the new methods of artificial insemination.

4. The number of fertilized ova which develop properly up to the time of birth is affected by several factors. Not only do they fail to become attached to the uterus

under conditions where progestin secretion from the corpus luteum is insufficient, but their absorption or abortion may be induced if deficiencies occur at a later stage of gestation. Foetal atrophy may be caused by genetic lethal factors in the embryo or by inherited maternal characters.

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THE INTERRELATIONSHIPS OF PIGMENTED AND COLOURLESS FLAGELLATA

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I. DISTRIBUTION OF COLOURLESS FLAGELLATA IN THE SYSTEM OF LIVING ORGANISMS

FLAGELLATA are unicellular organisms which swim by means of one or several flagella. In this respect they resemble many species of bacteria, from which, however, they differ in their considerably larger size and higher cell differentiation, and above all in the possession of a nucleus.

The Flagellata do not constitute a natural systematic unit. They belong to quite different branches of the kingdom of living organisms, within which they constitute rather a stage of organization than a phylogenetically delimited group. There is much in favour of the view that the Flagellata represent the most primitive stage of present-day organization within the different branches, in spite of their often highly complicated structure.

An approximately parallel development of the different branches or series is most distinctly shown in Chrysophyceae, Dinophyceae, Heterokontae and Chlorophyceae, but nearly all species of Euglenophyceae and Cryptophyceae likewise possess a flagellate organization (cf. Fritsch, 1929[1]).

The recognition of the fact that what are called Flagellata are systematically so heterogeneous makes it all the more remarkable that each of the larger groups includes both pigmented and non-pigmented species. The presence of pigment is of course related to the mode of nutrition, which as a general rule has a greater influence on organization than any other biological function.

Most of the pigmented Flagellata are green, but even when they appear brown, blue or red, chlorophyll is always present, associated with carotenoids and sometimes with other pigments as well. All these pigments are confined to distinct parts of the protoplasm, called chromatophores, or better, plastids. Chlorophyll, and probably

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also the accessory pigments, function in carbon assimilation. They are therefore grouped as assimilatory pigments in contrast to occasional other pigments which show a different distribution.

Higher plants decompose carbon dioxide within their chloroplasts with the help of the radiant energy of the sun, producing carbon compounds which serve as building materials and as a source of energy. If deprived of the possibility of carrying out this process by the absence of carbon dioxide or of light they must starve. The same is true of the chlorophyll-containing Flagellata which are dependent on carbon assimilation. All organisms lacking chlorophyll live on carbon compounds built up by chlorophyll-containing ones, although the way in which this is accomplished differs in bacteria, fungi and animals.

A similar profound difference in nutrition, due to the presence or absence of assimilatory pigments, exists also within those series of Flagellata which include both coloured and colourless representatives. Leaving out of consideration the artificial systematic groups which have been established as a provisional means of classifying the numerous colourless Flagellata, the other orders, namely, the Chrysomonadineae, Cryptomonadineae, Dinomonadineae, Euglenomonadineae and Phytomonadineae, comprise many pigmented and comparatively few unpigmented species. A similar relation obtains among the unicellular chlorococcales and diatoms, where, however, colourless species are still more scantily represented.

The unpigmented forms are often readily recognized as relatives of pigmented species, although sometimes this presents difficulties. If the forms in question are Algae they may be mistaken for Fungi, whilst if they are Flagellata they will be referred to the completely colourless orders if their systematic position cannot be established. This is of course merely a provisional resource. The line of demarcation from those orders whose species are for the most part pigmented has already shifted and may undergo further displacement as new means of differentiation are discovered. It does not, however, appear that we shall ever be in a position to abolish the orders of colourless Flagellata and to refer all their members to their true affinities. Many of them have undergone too much modification, and their organization has, in the course of a long evolution, become far removed from that of their coloured ancestors.

The fact that nearly all orders of pigmented Flagellata include representatives devoid of pigments has been established by morphologists who have hitherto been almost alone in this field of research. There are few short comprehensive reviews of the subject and not one of them is complete. Pascher (1927, 1931) has collected much valuable material relating to Phytomonadineae and has dealt with it in an ingenious manner. Of the equally interesting colourless Euglenomonadineae not much has been heard since the classical work of Klebs (1883, 1892) even up to the present day. The literature on unpigmented Dinoflagellata or Peridineae is very scattered. The monograph by Schiller (1933) and a recent paper by Harris (1940) are instructive. It is clear, however, that the colourless relatives of coloured species arose by loss of pigments or apochlorosis (apochromasis) and not in the reverse way as Dangeard still held in 1901.

SYNOPSIS OF THE APOCHLOROTIC FLAGELLATA AND OF THEIR NEAREST RELATIVES AMONG PIGMENTED SPECIES

CHRYSOMONADINEAE

Pigmented	Unpigmented
Chromulina	*Oicomonas ocellata* Scherffel = *Heterochromulina ocellata* (Scherffel) Pascher
Ochromonas	*Monas vivipara* Ehrenberg = *Heterochromonas vivipara* (Ehrenberg) Pascher
Uroglena or *Uroglenopsis*	*Monas sociabilis* H. Meyer
Mallomonas	*Mallomonas apochromatica* Conrad. Species of *Monas*, *Stokesiella*, *Physomonas* and other Monadaceae hitherto classed among Protomastigineae probably all belong here, perhaps also *Spongomonas*

CRYPTOMONADINEAE

Cryptomonas, *Chroomonas* and others	*Chilomonas paramecium* Ehrenberg *Cyathomonas truncata* Fresenius

DINOMONADINEAE

Amphidinium, *Gymnodinium* and others	*Amphidinium Elenkini* Skvortzow has very small or no chromatophores *Gymnodinium helicocoster* Harris is sometimes colourless and saprophytic

The following species and many others are devoid of pigments and probably holozoic:

Gymnodinium blax
G. colymbeticum
G. fungiforme
G. helveticum
G. vorticella
Glenodinium edaxi
Massartia campylops
Spirodinium hyalinum

PHYTOMONADINEAE

Dunaliella	*Hyaliella polytomoides* Pascher
Tetrachloris (perhaps also *Pyramidomonas*)	*Polytomella agilis* Aragao *P. aphanochloris* Skuja *P. caeca* Pringsheim *P. citri* Kater *P. Dofleinii* Pringsheim
Chlamydomonas	*Polytoma acutum* Pringsheim *P. caudatum* Korshikov *P. ocellatum* Francé *P. uvella* Ehrenberg and many others *Tussetia polytomoides* Pascher
Chlorogonium	*Hyalogonium acus* Pascher *H. Klebsii* Pascher
Coccomonas	*Chlamydoblepharis brunnea* Francé (uncertain)
Furcilia	*Furcilia lobosa* Stokes
?	*Collodictyon triciliatum* Carter
?	*Aulacomonas submarina* Skuja
Brachiomonas	*Hyalobrachion omphalotus* Swindell
Carteria	*Tetrablepharis globulosa* Senn *T. multifilis* (Klebs) Wille *T. obovalis* Pascher

EUGLENOMONADINEAE

Genera

Euglena	*Astasia*
Eutreptiella	*Distigma*

Genera with unpigmented species and species with unpigmented varieties

Euglena acus (aggregate species)	Here belong a number of apochlorotic parallel forms, e.g. *Euglena acus* f. *hyalina* Klebs, 1883 *Cyclidiopsis acus* Korshikov, 1917 *Menoidium acutissimum* Playfair, 1921 *Euglena acus* var. *pallida* P. Dangeard, 1930 *Cyclidiopsis Korschikoffii* Matvienko, 1938 *Astasia linealis* Pringsheim, 1938 *Cyclidiopsis acus* Korshikov, Skuja, 1939 *Euglena acus* f. *hyalina* Klebs, Skuja, 1939
Euglena viridis	*Euglena viridis* f. *hyalina* Klebs, 1883; rediscovered Pringsheim, 1940
Euglena gracilis	Hyaline light-form Ternetz, 1912; again obtained by Pringsheim, 1940 A considerable number of, in part, insufficiently described species of colourless Euglenae or of *Astasias* with stigmata show more or less similarity with *E. gracilis*: *Astasia ocellata* Khawkine, 1885 *Euglena quartana* Moroff, 1904 *Astasia quartana* Pringsheim, 1936 *Khawkinea halli* Jahn & Kibben, 1937 Four further different strains are in culture:
Lepocinclis	*Gyropaigne kosmos* Skuja
Phacus	*Phacus triqueter* var. *hyalina* Perty *P. pleuronectes* f. *hyalina* Klebs = *Hyalophacus Klebsii* Pringsheim *H. ocellata* Pringsheim
Trachelomonas	*Trachelomonas reticulata* Klebs *T. volvocina* var. *hyalina* Klebs *T. obovata* var. *Klebsiana* Deflandre

II. REFERENCE OF COLOURLESS FORMS TO GROUPS OF PIGMENTED ORGANISMS

It follows from this synopsis that the apochlorotic Flagellata, in spite of their wide distribution, are nevertheless represented only by a small number of species scattered over the various systematic groups. The generic names vary to a striking extent because reference of these forms can be carried out from different points of view. If the relationship is obvious, the form concerned is referred to a definite genus or even species. If such an allocation is impossible, special groups of unpigmented monads must of course be created. Thus the Peranemaceae, which are not enumerated above, are still readily recognized as Euglenineae both by their cellular organization and by the presence of paramylon, a starch-like reserve substance, although no closely related green species or genera are known. This is related to the fact that the Peranemaceae have undergone appreciable change in organization since their separation from the green Euglenineae as a result of their holozoic nutrition. This change is less appreciable in the saprophytic Astasiaceae which are still closely related to the Euglenaceae, so that the genus *Astasia* itself can be regarded as the apochlorotic parallel of *Euglena* so long as no more profound differences than those hitherto known are discovered. Among Astasiaceae there are, however, genera such as *Menoidium* and *Rhabdomonas*, for which no near parallel can be found among the green Euglenaceae, although it is obvious that they are more closely related to the genera *Phacus* and *Lepocinclis* than to *Euglena* in the possession of a rigid body.

The features, by means of which actual relationship can be recognized, are those customary in taxonomy, that is almost exclusively morphological ones. It is, however, as stated above, difficult to establish rules on which nomenclature can be based. Types which probably have about the same degree of relationship to green species have in part been regarded as varieties and in part referred to independent species, genera, families or even orders. That depends not only on the opinion of the individual investigator, but above all on the loss or retention of characteristic features going hand in hand with the absence or presence of pigments. Since we are dealing with unicellular organisms, the number of persisting features is sometimes markedly limited.

This may go so far that forms devoid of pigments betray little or nothing of their relationships. In the latter case they do not come within the province of these considerations, but belong to the large host of colourless Flagellata which are usually arranged on quite different principles. We must, however, not forget that previously this applied to all colourless Flagellata and that the line of demarcation is ill defined, and is bound always to remain so. Species which could not hitherto be assigned to any group may, by taking into consideration new features, prove to be chrysomonads, dinomonads, etc., which have lost their pigments.

The recognition of colourless flagellates as relatives of pigmented ones is in certain instances easy. If, for example, we find a flagellate with a rounded envelope, a single long flagellum, and glistening granules which do not stain with iodine in the interior, as well as the vacuolar system of Euglenineae, there will be no doubt that we are concerned with a member of the genus *Trachelomonas*, notwithstanding that green chromatophores are lacking. The structure of the envelope may even render reference to a definite species possible. In such a case we follow Klebs in speaking of it as a hyaline variety or "forma". This is so in *Trachelomonas volvocina* f. *hyalina* Klebs, while *Trachelomonas reticulata* has hitherto not been found in a green state. In other Flagellata possessing no sculptured envelope the reference would be much more difficult.

If *Polytoma* be the organism concerned, investigation of the reproduction will be necessary in order to recognize that we are dealing with a chlamydomonad devoid of chlorophyll. Such organisms can still be referred to a definite genus but this is no longer easy. It is even harder to recognize *Astasia* as a colourless *Euglena*, or *Distigma* as a colourless *Eutreptiella*, whilst in the case of *Menoidium* and *Rhabdomonas*, as already stated, an exact allocation is no longer possible. We can only determine the family.

The reserve substances commonly afford an especially handy indication of affinity. Starch, which is characteristic of Peridineae, Cryptomonadineae and Phytomonadineae, is also found in the apochlorotic members, e.g. *Gymnodinium helicocoster*, *Chilomonas paramecium*, and species of *Polytomella*, *Polytoma*, *Hyalogonium*, *Tetrablepharis*, etc. Paramylon is found both in colourless and in green Euglenineae.

Since in pigmented species these reserves are connected with carbon assimilation, it is not astonishing that they have been lost in more highly derived forms like *Cyathomonas* among cryptomonads or *Tussetia* among phytomonads. It is all the more significant that they are still produced by a large number of species after loss of assimilatory pigments. They thus afford an indication of near relationship to coloured species.

Similar considerations apply to the eye-spot or stigma. It is doubtful whether this structure is in any way phylogenetically connected with the primitive eyes or ocelli of animals. Stigmata are widely distributed among chlorophyll-containing Flagellata, and obviously play a role in phototaxis which serves to bring the cells into conditions of illumination suitable for carbon assimilation. Colourless Flagellata with saprophytic or holozoic nutrition usually possess no stigma, although there are a number of such forms in which one is present. All of these are phototactic, in contradistinction to those without an eye-spot. Colourless Flagellata possessed of an eye-spot are found among chrysomonads, dinomonads, phytomonads and euglenomonads, in short in all series the members of which are usually coloured (Pringsheim, 1937*c*). The only exception is constituted by the cryptomonads, among which even the pigmented types mostly lack a stigma and among which only one saprophytic species is known. The presence of a stigma reveals a close relationship to chlorophyll-containing forms (Pringsheim, 1937*c*, p. 239).

Other features warranting the conclusion of a near relationship between colourless and coloured organisms are afforded by the entire structure of the cell-body, as well as by the organs of locomotion. Thus the apochlorotic Phytomonadineae, in addition to having a similar type of reproduction, both sexual and asexual, to the green forms, have a similar cellular envelope and shape, as well as a comparable structure of the nucleus and of the flagellar apparatus. The two or four flagella are whip-like with basal blepharoplasts. Colourless euglenoids, on the other hand, share the ciliate flagellum with the green forms. Among Chrysomonadineae the cyst with its silicified envelope, pore and stopper provides a common feature. The flagella, which have as yet only been investigated in a few species, are, like those of euglenoids, beset throughout their length with cilia which may likewise come to be of value in deciding the relationship of a colourless form in doubtful cases (Vlk, 1938).

The feature which appears as of greatest importance in all the discoveries and problems connected with the phenomenon of apochlorosis is the often extremely close relationship between organisms genotypically devoid of chlorophyll and the chlorophyll-containing ones; the latter are obviously vegetable in nature. It is one of the merits of Ferdinand Cohn (1854), best known as the founder of scientific bacteriology, to have been the first to realize this. He quite justifiably referred the colourless *Polytoma uvella* Ehrenberg as *Chlamydomonas hyalina* to the phytomonads and was also the first to describe unpigmented diatoms. He correctly recognized the biological importance of both as inhabitants of putrefying materials.

Thirty years later Klebs (1883) approached even more closely to the question by referring colourless euglenoids as "forms" to definite species, namely *Euglena* *acus*, *E. sanguinea* and *E. viridis*. In *E. acus* he discovered two hyaline varieties, one with and one without a stigma. Klebs observed a hyaline variety of *Phacus pleuronectes* without a stigma, and I subsequently found another possessed of a stigma. In these instances there are presumably several other differences between the varieties with and without an eye-spot, although these differences need not go beyond the usual boundaries of a variety. There are, however, other forms with and without a stigma which can scarcely be differentiated on the basis of other features or only after a very detailed investigation. This is so in *Polytomella*, *Hyalogonium*, *Tetrablepharis* and *Astasia* and most strikingly in *Polytoma*.

III. THE PHYSIOLOGICAL ASPECTS OF APOCHLOROSIS

It becomes more and more obvious that these affinities cannot be satisfactorily elucidated by mere microscopical investigation of occasional material. For this purpose we need cultures and physiological research. It is only when the morphological-systematic and the phylogenetic points of view are combined with physiological and ecological ones to form a complete picture that the many important problems in this field of research become plain. Some progress in this direction has been made during the past twenty years since the time when, on the basis of ecological considerations and the first successful pure cultures, it was shown that *Polytoma* is an acetate organism (Pringsheim, 1920, 1921). Lwoff and his pupils contributed other examples of a similar trophic nature and have summarized all the conclusions reached up to date as to the nutrition of apochlorotic Flagellata (Lwoff, 1932; Provasoli, 1937/8). The multiplication of all these species of apochlorotic Flagellata, as well as of the green ones capable of saprophytic nutrition, which have so far been grown in pure culture—about twenty in all—is without exception markedly promoted by acetates. The effect of other lower fatty acids and of certain other organic compounds which are utilized by some of the species is diverse. *Chilomonas*, for example, and certain *Astasias* will multiply if provided with peptone alone, while this is not so in the Phytomonadineae, such as species of *Polytoma*, *Hyalogonium* and *Polytomella*. Sugars and the higher sugar alcohols are not utilized by any of the species mentioned, although carbohydrates are deposited as reserve substances in the cells.

All advances in our knowledge beyond mere description are due to the improvement of culture methods. All apochlorotic Flagellata can be caused to multiply in artificial media, although up to the present it has not been possible in every instance to do so after excluding bacteria. By raising thousands of individuals from a single cell the morphological features can be adequately gauged, living material for physiological investigation is available and a provisional insight into the conditions of life and of multiplication is obtained.

It must be specially emphasized that without cultures even the taxonomy of Flagellata must remain in its earliest stages, as is obviously true of the physiology of these organisms. In many instances specific description is only possible if homogeneous material derived from a single cell is assured and can be examined under diverse conditions. Only with the aid of cultures can adequate material be obtained for detailed cytological investigation. It is also of special importance to be able to compare a newly found form in a living state with those that have been previously observed. It is therefore of great importance to keep permanent cultures. The most characteristic features of organisms are often no longer recognizable in fixed material. A culture can usually be initiated from even one or from a few cells, which would be insufficient for an adequate specific description. I hope that a similar technique may soon become available for the study of the difficult biology and taxonomy of the Ciliata, for culture methods analogous to those applied to flagellates can presumably be utilized for many ciliates.

Culture methods involve in the first place a close imitation of natural conditions based on observation of material from the natural habitat and of ecological and biochemical considerations. If water containing flagellates and ciliates is brought into the laboratory, the composition of the medium changes rapidly and the specific nutrient substances are consumed. The species originally present are starved or displaced by others. If, however, bottom-mud from the water concerned is added, development usually proceeds and the organisms remain healthy for a longer time. This is especially the case if the mud contains decaying organic remnants in sufficient quantity and of the right quality. These are decomposed by anaerobic bacteria and transformed into soluble organic compounds which either serve directly as nutrients for the flagellates and ciliates living in the overlying water or indirectly via the bodies of bacteria.

Similar conditions are readily reproduced even in the confined space of a small test-tube. Garden soil is used to provide a mud. This is distributed over a small quantity of a suitable putrefiable substance, such as plant seeds, albumen, starch, etc. Water is added to cover the "artificial mud" to a depth of 5–8 cm. and the whole is heated for half an hour in a steam sterilizer. The resistant spores of the soil bacteria remain alive or are subsequently introduced into the culture. If cells of a given species of flagellates are thereupon introduced, they mostly develop very abundantly after a certain latent period. Under some circumstances the development of a harmful acidity must be prevented by the addition of calcium carbonate. In this way thousands of genetically homogeneous individuals can be raised from a single cell picked up in a capillary pipette (Pringsheim, 1936, pp. 46 *et seq.*). Phytomonadineae and Euglenineae nearly always afford successful cultures by this procedure, and in most cases also Cryptomonadineae and Chrysomonadineae, but with dinoflagellates no success has so far been obtained.

For exact breeding experiments, and especially for the investigation of the physiology of nutrition, pure cultures devoid of bacteria are necessary. These afford no difficulties with the Phytomonadineae, having been first achieved with *Polytoma* (Ogata, 1893; Pringsheim, 1920, 1921). Numerous species and strains of this genus have since been reared in pure culture, and likewise single species of the rare genera *Hyalogonium* (Pringsheim, 1937 *a*) and *Polytomella* (Pringsheim, 1937 *b*), as well as of the saprophytic genus of Cryptomonadineae, *Chilomonas* (Pringsheim, 1921; Glaser & Coria, 1930; Loefer, 1934; Pringsheim, 1934). On the other hand, no pure cultures of dinoflagellates and chrysomonads have at present been obtained, while among Euglenineae, despite numerous attempts, only a few species have been successfully grown free of bacteria.

The technique for obtaining pure cultures of saprophytic Flagellata has only recently been developed. The old bacteriological plating method with agar media was successful only with species of *Polytoma.* Except in this genus it has not so far been possible to rear any species on agar. In certain species of *Polytoma,* and in some eight to ten other saprophytic flagellates, another method of purification has proved successful, namely the technique of pipetting or washing. This consists in the transference of single cells by means of fine capillary tubes from one drop of sterile liquid medium to another until they are freed from all bacteria or other micro-organisms. The operation usually succeeds without undue trouble, especially if the species concerned can withstand slightly acid solutions, in which most bacteria cannot thrive. The main difficulty in obtaining pure cultures of flagellates does not therefore lie in the process of purification itself, but in finding the conditions under which multiplication of individuals takes place.

The occurrence of these forms in nature is of course chiefly related to their nutrition. Where remains of animals and plants are undergoing decay, products of protein-decomposition are formed which constitute the best sources of nitrogen for colourless saprophytic Flagellata. During the decomposition of proteins, fats and carbohydrates (e.g. starch and cellulose) under anaërobic conditions lower fatty acids originate. These processes take place especially in the bottom deposits of stagnant waters, and from here the compounds in question diffuse into the overlying water and serve as nutrient substances for the flagellates.

IV. ORIGIN OF COLOURLESS FLAGELLATA

The phylogenetic origin of apochlorotic from chlorophyll-containing Flagellata offers various problems, both of a morphological and of a physiological nature. For example, the question arises as to whether after loss of the assimilatory pigments the protoplasmic stromata of the plastids persist. The pigmented chromatophores might possibly change into colourless leucoplasts. The latter are difficult to recognize and no method of staining seems to afford an indubitable proof of the nature of the cell-organs in question.

Loss of pigments may be supposed to have taken place in different ways, either as a gradual paling or a diminution in size of the chromatophores to the point of disappearance, or, on the other hand, by a degeneration of these structures. Moreover, complete loss of chromatophores might result from a disturbance of the correlation between multiplication of cells and of chromatophores. This would upset the rhythm of division. Normally each chromatophore divides on an average once during the interval between two cell divisions, thus maintaining approximately the same number of chromatophores in the cells. If, however, the chromatophores divide more slowly than the cells, a gradual diminution in the number of plastids will take place which may even go so far as to result in their complete disappearance in individual cells.

There are some data to indicate the occasional occurrence of each of these modes in the evolution of apochlorotic cells. The possibility of the origin of colourless cells as a result of morphological changes does not explain however the existence in nature of viable apochlorotic descendants of pigmented species. The loss of the organs or pigments needful for carbon assimilation must lead to starvation if the species in question is dependent for its food-supply on the reduction of carbon dioxide. Colourless species must therefore be capable of utilizing organic substances available in the environment, but they cannot adopt this mode of nutrition the moment they have lost their assimilatory pigments. It is obvious that the capacity to do so must have existed previously.

This means that apochlorotic forms may be expected to occur only in those taxonomic groups the members of which usually exhibit a mixotrophic mode of nutrition, an hypothesis which is capable of being put to the test. In this respect there are not as many certainly established data as are desirable, since hitherto only thirteen species of colourless Flagellata have been raised in pure culture and not many green ones have been sufficiently investigated as to their ability to utilize organic compounds. But so far as our knowledge goes the postulate expressed above conforms with actual facts.

In order to investigate the ability to utilize organic compounds, pure cultures must be available and there must be some preconception as to the kinds of nutritive substances that come into question. If green, or more accurately chlorophyll-containing, organisms are concerned, carbon assimilation must be excluded, and the simplest way of doing this is to place the material in the dark. All unicellular pigmented algae that have hitherto been tested have been brought to multiply in the dark when suitable nutriment was provided. This is true of Ulotrichaceae, and likewise of Chlorococcaceae and their allies the Chlamydomonadineae. Whilst, however, among Ulotrichaceae and Protococcaceae (*Coccomyxa*, *Chlorella*, *Scenedesmus*, *Hormidium*, *Stichococcus*, etc.), as well as in the colourless *Prototheca*, sugar is just as suitable as acetate for maintaining multiplication in the dark, the green Chlamydomonadaceae (*Chlamydomonas*, *Chlorogonium*, *Haematococcus*) only make use of fatty acids (Lwoff & Lwoff, 1929; Lucksch, 1932; Pringsheim, 1937 *a*). In this respect therefore they behave just like their colourless allies *Polytomella*, *Polytoma* and *Hyalogonium*.

No experiments with pigmented cryptomonads have previously been undertaken, but I have recently found a species in moorland water that utilizes acetate and peptone, in the same way as its colourless relative *Chilomonas*.

The euglenomonads occupy a peculiar position. Among the green species only a few have been raised in pure culture and the physiology of their nutrition examined. Of these only one, the frequently mentioned *Euglena gracilis*, is able to multiply in the dark. It also constitutes a remarkable exception in producing no chlorophyll when deprived of light. Elsewhere this has only been established in angiosperms and in moss protonemata (Pringsheim & Pringsheim, 1936). *E. gracilis*, when cultivated in the dark, is therefore very similar to a colourless *Astasia*. In order to multiply in the absence of carbon assimilation it needs peptone and lower fatty acids, acetic acid being the best. In the physiology of its nutrition it therefore again behaves like those species of Astasiaceae of which pure cultures have been obtained.

Euglena gracilis is also remarkable in another respect. It serves to show one of the ways in which loss of pigments may be presumed to have occurred in the evolution of apochlorotic Flagellata. We are not here concerned with the common colourless, or rather yellowish, form grown in the dark, which becomes green within a few hours after exposure to light. Such modifying influences do not persist. Ternetz (1912) has, however, described an "intermediate form" of *E. gracilis*, the chromatophores of which degenerate under certain conditions so that single, completely colourless, cells devoid of plastids arise. Their descendants never become green again. Even the eye-spot may be lost, so that the apochlorotic race is indistinguishable from a "true" *Astasia*. Ternetz further occasionally found quite similar colourless individuals, likewise without an eye-spot, which are stated to have originated by a disturbance in the harmony of the division-process.[1]

After a long-continued search, a strain of *Euglena gracilis* was found, similar in behaviour to the intermediate form of Ternetz. Although it still requires a fuller study certain results are already available. Under certain circumstances there appear in clone cultures with organic substances individuals which are completely colourless, and not yellowish like the hyaline form grown in the dark. They possess a stigma which may however be smaller than that of the typical species. From single colourless cells one can obtain sub-clones, in part green, in part behaving like the primary clone, that is they segregate again. Microscopical investigation exhibits degenerating chromatophores which at first still appear green, then they become yellowish and gradually vanish with the appearance of red granules. Although occasional individuals with only two, or even a single, chromatophore are found, there is nothing to justify the conclusion that such have arisen through a disturbance in the harmony of cell division. Permanent colourless sub-clones were obtained.

The possibility that disturbances in cell division might lead to the appearance of apochromatic races has so far not been further supported by facts. From a theoretical standpoint, however, this seems plausible and casual observations of various biologists speak in favour of the occurrence of single cells devoid of chromatophores. Apart from degeneration, such as occurs in *Euglena gracilis* var. *Zumsteinii* (Lwoff, 1932), a gradual reduction in the size of the chromatophores must be regarded as altogether likely. Among Phytomonadineae there are certain species, with remarkably small chromatophores (Pascher, 1927; Skuja, 1927). It is not known whether their photosynthetic capacities suffice for autotrophic nutrition.

It also remains dubious whether persistence of the stroma of the plastids, despite disappearance of the pigments, can be assumed in any instance. Volkonsky (1930) in *Polytoma* and P. Dangeard in *Euglena acus* var. *pallida* (1930) have indeed rendered visible, by means of staining, certain structures which they regard as colourless plastids, but there are facts which speak against this interpretation. Recently, I have found an *Astasia* or colourless *Euglena* with a stigma and possessed of a shape similar to that of *E. viridis*. Klebs (1883) described a hyaline variety of this species, and I had wondered how he could have recognized it as a variety of *E. viridis* in the absence of the only characteristic feature, viz. the star-shaped chromatophore with a central pyrenoid. Klebs has, however, again proved to be right, as in so many other cases. My apochlorotic variety possesses not only the same body shape, but also shares the same arrangement of protoplasm and distribution of paramylon grains as in the green parent form so that the affinity is apparent. Plastids and pyrenoids were, however, sought in vain.

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[1] This excellent and much cited paper by Ternetz is unfortunately open to certain doubts. Single cells fail to multiply in pure culture in any of the media used. It also appears strange that apochlorotic cells should develop in two different ways, nor does either mode explain the disappearance of the eye-spot. Other investigators have been unable to observe phenomena like those described by Ternetz in any strain of *Euglena gracilis*. I therefore in 1914 drew the conclusion that the strain examined by Zumstein and Ternetz must behave differently from the majority of other strains of *E. gracilis*. It was called var. *Zumsteinii* by Lwoff.

Six different unpigmented Euglenas provided with a stigma are now in culture and it is hoped that their detailed investigation will throw some more light on the origin of colourless races from pigmented species.

It may not be going too far to say that the apochlorotic Flagellata are in certain ways unique among living organisms. They afford a clear example of affinity with forms which differ from them so markedly in their mode of nutrition, a difference induced by the loss of the assimilatory pigments. Green and colourless varieties are known within the limits of a sharply defined species. It is even possible to follow in a test-tube one mode of transformation which may have occurred in the ancestry, namely by degeneration of plastids. This change proves to be permanent and irreversible though not of the nature of a mutation in the commonly accepted sense. Moreover, it is noteworthy that some colourless forms (e.g. *Chilomonas paramecium* and *Polytoma uvella*) occur in large numbers in nature. Finally there is the repeated appearance of the phenomenon of apochlorosis in numerous species belonging to groups of diverse parentage. It must be remembered in this connection that, for example, Chrysomonadineae and Phytomonadineae are systematically very remote from one another, and that similar instances are to be found among Cyanophyceae, Diatomeae and Chlorococcales. These facts render the apochlorotic Flagellata of great importance for the investigation of biological problems which are not so readily studied in other living organisms.

V. SUMMARY

1. The Flagellata do not constitute a natural taxonomic group, but represent diverse branches of the kingdom of living organisms. Certain orders of colourless Flagellata are quite artificial, while the majority comprise large numbers of pigmented and a few non-pigmented forms; some of the latter are closely related to certain pigmented species.

2. Certain colourless forms are readily linked up with their pigmented relatives, but in other instances scarcely any indications of their affinities are to be found. The means of recognizing affinities are afforded by cellular morphology, methods of reproduction, resting stages, types of food reserves present, occurrence of eyespots, etc.

3. Cultures are indispensable for the modern study of micro-organisms. They provide homogeneous material for morphological and physiological investigation. For experiments on nutrition pure cultures are essential. So far as such experiments have been performed they show that pigmented and related non-pigmented forms require similar kinds of nutriment.

4. Loss of pigments seems always to be accompanied by loss of plastids. This may result from gradual reduction in number or in size of the plastids. Degeneration of plastids has already been described by Ternetz in a certain strain of *Euglena gracilis*. A similar strain has been found again and completely colourless races have been raised. Other examples of apochlorosis have not been so fully investigated. It remains doubtful whether reduction in the amount of pigments can go so far as to give rise to colourless forms.

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THE GAS-VACUOLES OF THE MYXOPHYCEAE (CYANOPHYCEAE)

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I. INTRODUCTION

The protoplast in many blue-green algae (Myxophyceae) is characterized at times by the presence of small structures of irregular shape presenting under high magnification very much the same appearance as air bubbles. The nature and significance of these structures, which have been variously termed gas-vacuoles (Klebahn, 1895), suspensory bodies (Molisch, 1903) and pseudo-vacuoles (Lemmermann, 1910), have been the subject of much speculation and discussion. The available evidence indicates that they are, in all probability, spaces in the protoplasm containing gas: the phenomenon is therefore of considerable general physiological interest. Since they are especially typical of the so-called "water-bloom" algae, which constitute a great nuisance in certain types of fresh water, the problem of the nature of the factors causing their formation is one of some economic importance. In the following critical review of our present knowledge of the subject the term gas-vacuole will be used.

II. THE DISTRIBUTION OF GAS-VACUOLES IN NATURE

Although especially characteristic of the Myxophyceae, the occurrence of gaseous inclusions in the cell is also recorded in other groups of living organisms. Thus gas-vacuoles, apparently of the same type, are found in some bacteria, e.g. *Thiothrix* (where they occur in addition to drops of sulphur) and *Sarcina ventriculi* (Kolkwitz, 1928). Among protozoa there are two genera, *Arcella* and *Difflugia*, in which gas bubbles are frequently present, but these differ from the gas-vacuoles of Myxophyceae in their spherical form and temporary character (Klebahn, 1922, p. 541).

The following genera of Myxophyceae include species specially characterized by the possession of gas-vacuoles:

Chroococcales: *Coelosphaerium*, *Microcystis*;

Hormogoneales: *Oscillatoria*, *Lyngbya*, *Nostoc*, *Anabaena*, *Anabaenopsis*, *Aphanizomenon*, *Gloeotrichia*, *Calothrix*.

Most of the algae concerned are planktonic or mud-inhabiting forms and in a considerable number of species the gas-vacuoles are sporadic in their occurrence. They have not been reported in any member of the Chamaesiphonales.

Gaseous inclusions appear to be entirely absent from the protoplasm of forms belonging to other groups of the plant and animal kingdoms.

III. THE CHARACTERISTICS OF GAS-VACUOLES

The structures in question are very minute, generally not exceeding 2μ in diameter. They are very irregular in shape but never possess acute angles. Van Goor (1918), however, records perfectly spherical gas-vacuoles in *Oscillatoria guttulata*. Although they often appear to be scattered throughout the chromatoplasm (peripheral zone of cytoplasm containing photosynthetic pigments) and centroplasm ("central body", possibly a rudimentary nucleus) of the cell, careful examination shows that they occur in a layer of the chromatoplasm bordering on the centroplasm (Geitler, 1936). In many species of *Oscillatoria*, *Lyngbya*, and *Phormidium* the gas-vacuoles occupy characteristic positions adjacent to the transverse septa.

(1) *Optical characteristics*

Viewed macroscopically, material containing gas-vacuoles is pale green or whitish by reflected light, dark and opaque by transmitted light. When the gas-vacuoles are destroyed by pressure (see p. 208) this appearance immediately alters, the mass becoming darker green and translucent. Under the microscope the individual gas-vacuoles appear somewhat reddish with wide black margins, offering a great contrast to the surrounding cytoplasm. Except for the red colour, the appearance is exactly like that presented by small inclusions of a substance of low refractive index within a medium of higher refractive index. Double refraction is not shown (Molisch, 1903).

(2) *Specific gravity*

When a plankton sample is shaken and then allowed to stand, the blue-green algae present soon collect at the surface of the water while most other planktonts tend to sink to the bottom. This clearly demonstrates that the blue-green algae have a specific gravity less than that of water. The fact that these algae almost invariably possess gas-vacuoles has led to the generally accepted view that the structures in question enable the alga to float. This has been questioned by Brand (1901) who pointed out that many sedentary blue-green algae possess gas-vacuoles and that he had observed *Anabaena flos-aquae* without gas-vacuoles floating at the surface. Lauterborn (1915) also stressed the fact that sedentary algae may show numerous gas-vacuoles and concluded from this that the latter are not responsible for the floating of planktonic forms. Utermöhl (1925, p. 294) records that many species of mud-inhabiting Myxophyceae do in fact become planktonic occasionally and among these are some mentioned by Lauterborn. Filaments of such algae in a sample placed in a plankton chamber were found to rise to the surface of the water.

An experiment which demonstrates the connection of gas-vacuoles with the ability to float is described by Ahlborn (1895), Klebahn (1895) and Strodtmann (1895). Suitable material is placed in a thick-walled flask filled with water and closed with a tightly fitting cork so that no air bubbles are included. If the cork is driven in with a blow from a hammer, the alga at once changes its appearance and sinks. Microscopical investigation shows that the gas-vacuoles have been destroyed. Algal material freed from externally adherent bubbles of gas by means of reduced pressure continues to float.

Molisch (1903) and Klebahn (1922) have observed that gas-vacuoles isolated from the algae (see p. 209) float to the surface of the liquid in which they are contained. To isolate the vacuoles Molisch used a solution of potassium nitrate. As pointed out by Fischer (1905) this solution would have a specific gravity greater than that of water so that Molisch's observation cannot be taken as showing that gas-vacuoles are specifically lighter than water. Klebahn (1922), moreover, questions whether the objects isolated by Molisch really were gas-vacuoles (see p. 209).

Klebahn (1922, p. 546), using two different methods, determined the specific gravity of material of *Gloeotrichia echinulata* in which the gas-vacuoles had been destroyed by pressure. He arrived at a value of from 1·0067 to 1·0085. On this basis he calculated that, if the vacuoles contain a gas, they must occupy at least 0·7 % of the volume of the cell to enable the alga just to float in water. If, on the other hand, the structures in question were supposed to contain petrol ether (sp. gr. 0·67) or camphor (sp. gr. 0·99) then 2·1 or 70 % respectively of the weight of the alga must be attributed to them. The volume of the gas-vacuoles, expressed as a percentage of the total volume of the cell, is given by Klebahn (p. 208) as 0·8, which therefore supports the theory that the vacuoles are gaseous inclusions in the cell (see p. 210).

(3) *The behaviour of gas-vacuoles under pressure*

It has already been mentioned that sudden pressure destroys the gas-vacuoles. This is most readily demonstrated by mounting a little of the material on a slide and applying local pressure on the cover-glass by means of a needle. The experiment only succeeds if spores or masses of mucilage be absent. The cells subjected to such pressure become quite hyaline but they do not burst, nor is any granular matter extruded (see Klebahn, 1922, p. 558).

Klebahn (1922) employed both compressed gas and the centrifuge as a means of applying pressure in experiments with *Gloeotrichia echinulata*. Using the former means in conjunction with a specially constructed chamber suitable for observation under the high power of the microscope, he found that a sudden increase of pressure of 2 or 3 atm. was sufficient to destroy the gas-vacuoles. In the slender hair-like ends of the filaments the gas-vacuoles disappear more readily than elsewhere. The process of disappearance cannot be observed since the sudden change in pressure invariably alters the focus of the microscope. If the pressure is applied gradually, the gas-vacuoles frequently withstand a pressure of 9 atm. without change. When a vacuole is destroyed by slowly increasing pressure, it does not exhibit a gradual diminution in size as would a free gas bubble. Instead it remains approximately the same size as originally but gradually becomes fainter and offers less contrast to the surrounding cytoplasm.

Similar results were obtained when the centrifuge was used as a means of applying the pressure. The pressure at the bottom of a centrifuge cup is given by the expression

$$k=\frac{h}{g}\frac{4\pi^2}{t^2}\left[r-\frac{h}{2}\right],$$

where k is the pressure in g./sq.cm.; h, the height of the water column in the cup in cm.; g, the acceleration due to gravity in cm./sec./sec.; t, the revolution time and r, the distance in cm. of the bottom of the cup from the axis of rotation. A pressure of 4·139 atm. applied in this way destroyed gas-vacuoles in species of *Anabaena* and *Gloeotrichia*, while one of 2·681–2·447 atm. destroyed only a few, and one of 0·708–0·362 left them unaffected. In experiments with material of *Microcystis* (*Clathrocystis*) *aeruginosa* it was necessary to enclose it in a small glass tube open at one end in order to prevent its floating at the surface of the water in the centrifuge cup; even 4·139 atm. did not destroy the gas-vacuoles in this species.

When a suspension of *Gloeotrichia* in water was subjected to sufficient pressure to destroy the gas-vacuoles, a decrease in volume was observed proportional to the amount of alga present. In connection with both methods of applying pressure precautions were taken to avoid errors due to change in temperature or expansion of the glass vessels employed as a result of the increased pressure. The volume of the alga occupied by gas-vacuoles calculated on this basis is about 0·8 %.

Gas-vacuoles do not, however, disappear in a vacuum. Molisch (1903) placed living *Aphanizomenon* in a vacuum for 5 hr. without any appreciable alteration in floating capacity or reduction in the extent of gas-vacuoles.

(4) *The action of heat*

Dried algal material can be subjected to temperatures as high as 228° C. without change in the gas-vacuoles (Klebahn, 1922, p. 537). Boiling in water, on the other hand, destroys them after a time.

(5) *The isolation of gas-vacuoles*

If the algal material is softened by rotting or by chemical treatment (e.g. 4% potassium nitrate) gentle mechanical pressure will bring about disintegration and lead to the liberation of the gas-vacuoles (Chodat, 1896; Molisch, 1903; Klebahn, 1922, p. 543). According to Klebahn (1922), however, the objects isolated by Molisch were not gas-vacuoles, but droplets of protoplasm. Isolated gas-vacuoles preserve their characteristic appearance and irregular outline.

(6) *Chemical characteristics*

Gas-vacuoles give negative results with tests for substances normally found as inclusions in cells. Thus, Molisch (1903) concludes that gas-vacuoles are not free sulphur, protein, resin, fat or tannin. They are not stainable by any of the usual methods. Fixatives such as formaldehyde and 1% osmic acid do not affect the gas-vacuoles, which in material thus preserved show the same properties as in fresh material. They are, however, destroyed by a variety of reagents and this effect is definitely independent of the osmotic properties of the latter. Klebahn (1922, p. 552) summarises the action of a large number of chemical substances.

Saturated solutions of salts, such as copper sulphate, sodium chloride, potassium nitrate and sodium nitrate, are without effect, but a saturated solution of potash alum destroys gas-vacuoles rapidly. Dilute acids also destroy them rapidly but alkalies, even when concentrated, have a very much slower effect.

Many organic substances, such as phenol, thymol, chloral hydrate or alcohol, in strong aqueous solution bring about rapid destruction of the gas-vacuoles. It is, however, impossible to single out any particular group to which the effect might be ascribed. Very small traces of certain organic compounds, mostly fat solvents, are sufficient to destroy gas-vacuoles. Molisch (1903) described experiments with material contained in a hanging drop in a damp chamber into which a little chloroform, ether or similar substance had been introduced. The vapour of the latter that diffused into the water containing the alga was sufficient to destroy the gas-vacuoles. Klebahn (1922, p. 554) carried out similar experiments in which the alga was shaken up with water and a little of the substance, the effect of which was to be examined. Hydrocarbons, e.g. pentane and benzene, applied in this way caused gas-vacuoles to disappear within 24 hr.; the action of their chlorine derivatives was more rapid. Other organic compounds, e.g. carbon bisulphide, propyl alcohol, aniline and clove oil, acted extremely slowly or were without effect.

IV. THE NATURE OF GAS-VACUOLES

(1) *Historical review*

Richter (1894) held that the structures in question were drops of sulphur in a soft oily condition, an interpretation which has been rendered untenable by chemical investigation. The actual founders of the gas-vacuole theory were Strodtmann (1895) and Klebahn (1895). The former showed that gas-vacuoles were responsible for the flotation of planktonic blue-green algae and suggested that they were spaces in the protoplasm containing a gaseous product of metabolism. Klebahn emphasized that the low refractive index and specific gravity, and the destruction by certain chemicals and by pressure were all best explained on the assumption that these structures contained gas. Since he was unable to bring about their disappearance in a vacuum, he assumed that the gas was enclosed in a rigid membrane. Brand (1901) criticized this hypothesis on the grounds that the vacuoles do not disappear in a vacuum and that attempts to isolate the gas had failed, but he did not offer any alternative suggestions.

Molisch (1903) has been the most vigorous opponent of the gas-vacuole theory. He claimed to have isolated two kinds of "suspensory bodies", one in a mobile state containing a number of smaller globules in active Brownian movement, the other of a viscous nature and lacking the included particles. He assumed, therefore, that the structures under discussion are of a liquid or viscous nature, although no suggestions were offered as to their actual composition. The weakness of this theory is that it fails to account satisfactorily for their disappearance under pressure, which was supposed to bring about solution of the constituents of the suspensory bodies (see p. 211). Moreover, it is based on observations at the limits of microscopic vision, on objects which may not have been gas-vacuoles (see p. 208). Lauterborn (1915) supported the theory of Molisch.

Fischer (1905) regarded the vacuoles as an optical effect due to the anisotropy of a carbohydrate anabaenin. According to his view all members of the group should show such vacuoles under favourable conditions. The action of traces of hydrocarbons was ascribed to the breakdown of the anabaenin during cytolysis. The possibility of isolating the gas-vacuoles renders this theory untenable.

(2) *The gas-vacuole theory*

Klebahn's arguments and experiments (1922, 1925, 1929) in support of the gas-vacuole hypothesis may be summarized as follows:

(1) The fraction of the volume of the cell occupied by the vacuoles is about 0·8 % (see p. 208). Klebahn calculated the minimum amounts of various substances of low specific gravity which would be requisite to enable the alga to float (p. 207). If the contents were a gas, he obtained a value of 0·7 % by volume which agrees well with that actually occupied by vacuoles. On the other hand, if the vacuoles were solid or liquid inclusions, much larger quantities would be required. Tests for substances of low specific gravity (e.g. fats) give negative results.

(2) The destruction of the vacuoles by pressure with an accompanying decrease in volume of the alga is best explained on the assumption that they contain a gas. An optical effect might be affected by unequal pressure causing distortion of the cell structure, but this would not explain the disappearance of the vacuoles when pressure is applied uniformly in all directions, as when the alga is freely submerged in water. A solid or liquid might be caused to dissolve by increase in pressure, provided that the process of solution was accompanied by a decrease in volume; thus, for sodium chloride there is an increase in solubility in water of 2·2 % for an increase in pressure of 500 atmospheres. It is hardly credible, however, that such increase in solubility could account for an abrupt disappearance under a pressure of 2 or 3 atm. Since solids and liquids are almost incompressible, it is moreover unlikely that pressure, applied uniformly in all directions, would cause the rupture of a membrane which had acted as a barrier to the solution of the solid or liquid contents of the vacuole. The solid or liquid contents could hardly be expected to contract sufficiently to allow crumpling and consequent rupture of a membrane enclosing them. This would, however, be easily possible if the contents were gaseous.

(3) The appearance of the vacuoles is much like that characteristic of small air bubbles and the contents must therefore have a low refractive index compared with that of the surrounding cytoplasm. This appearance might, however, be due to a liquid or solid of very low refractive index.

(4) Klebahn in particular has carried out numerous and diverse experiments which show that more gas can be extracted from algae containing these vacuoles than from similar forms in which the latter have been destroyed and which have again returned to a state of equilibrium with the surrounding medium. This can be established under the microscope by mounting a normal colony of *Gloeotrichia echinulata* side by side with one from which the gas-vacuoles have been removed, in strong sulphuric acid. Numerous gas bubbles are evolved from the former but only very few from the latter.

Klebahn, using four different methods, has carried out experiments in which appreciable quantities of gas have been obtained from the same alga. The difficulties of this operation are considerable, since the vacuoles form only a small proportion of the material which itself contains much dissolved gas. The latter can be eliminated to some extent by various means, but complete elimination is impossible. The means employed for obtaining the gas consisted of (*a*) a modification of Preusse and Thimann's method with boiling water, (*b*) the destruction of the vacuoles by sudden pressure and removal of the gas by means of a vacuum, (*c*) the use of strong sulphuric acid and (*d*) destruction of the vacuoles by autoclaving, the gas being liberated with the help of a vacuum created automatically on cooling by an ingenious method (for details see Klebahn, 1922, 1925, 1929). Mean values obtained in a number of experiments conducted by the first method are:

	100 c.c. water yielded	1·84 c.c. gas
(*a*)	100 c.c. alga devoid of vacuoles yielded	1·55 c.c. gas
(*b*)	100 c.c. alga with vacuoles yielded ...	2·72 c.c. gas
	b – *a* (gas from vacuoles)	1·17 c.c.

The third and fourth methods gave mean values of 0·88 % and 0·86 % respectively for the extra volume of gas due to the presence of gas-vacuoles. These figures are in good agreement with the value of 0·8 % obtained by other means (see p. 208).

It seems unlikely that the additional gas obtained from algae containing gas-vacuoles could have originated from the decomposition of a solid or liquid occupying the vacuoles, since equivalent amounts of gas are obtained by methods involving such widely different means as heat, pressure and chemical action. It seems far more probable that this gas was actually present within the vacuoles.

In order to explain certain anomalous features in the behaviour of gas-vacuoles, Klebahn supposes them to be surrounded by a rigid impermeable membrane. The existence of such a membrane might account for the permanence of the vacuoles and their resistance to reduced pressure. It might also explain the retention of the irregular shape by isolated vacuoles. The action of certain reagents may be ascribed, not to their efficiency as gas absorbents, but to their action on the vacuole membrane, the absorption of the gas following automatically once the protecting membrane is removed. In the destruction of the vacuoles by pressure the membrane must first be ruptured by crumpling, minute cracks then allow water to be forced into contact with the gas. This would explain the peculiar manner in which the vacuoles are destroyed by slowly rising pressure (p. 208).

It is to be expected that protoplasm in contact with a gas would become profoundly modified, so that a bounding membrane would be formed. Klebahn argues that such a membrane could not be water-imbibed, since if that were so it would allow of diffusion of the gas and its consequent solution. Since the vacuoles are destroyed by traces of fat solvents, he suggests that the membrane is of a lipide nature (see p. 214).

Klebahn discusses on a theoretical basis the probable pressure of the gas within the vacuoles. The pressure inside an air bubble is given by the expression $d=2a/r$, where r is the radius and a the surface tension of the surrounding liquid (for water about 75 dynes per cm.). This gives the following values for small air bubbles in water:

Diameter of bubble in μ	Pressure in atmospheres
0·5	6
1·0	3
2·0	1·5
3·0	1

These values can only be applied partially to gas-vacuoles since, among other reasons, the latter are rarely spherical. For an irregular curved surface Laplace and Poisson give the general expression $d=a\left[\frac{1}{R}-\frac{1}{r}\right]$, in which R is the bigger and r the smaller radius. The values calculated with the help of this formula are smaller; for example:

R in μ	r in μ	d in atmospheres
∞	0·5	1·5
10	1·0	0·825

Moreover, in the case of the vacuoles, the surrounding medium is not water but protoplasm, for which Czapek has found the surface tension to be from 0·68 to 0·69 of that of water. Assuming that this value holds for the protoplasm of the Myxophyceae and especially for the membrane of the gas-vacuole, the above values would have to be diminished by roughly one-third. The turgor pressure of the cell, which in *Gloeotrichia echinulata* is 5 to 6 atm., must also be taken into account, since this will be transmitted inwards and will raise the pressure in the gas-vacuoles. Finally there is the surface tension arising at the surface of the protoplasm and directed inwards. For spherical water drops the size of *Gloeotrichia* cells (8–10μ) this pressure as given by the first formula is 0·3–0·37 atm.

Taking all the above data into consideration, a pressure of considerably more than an atmosphere must exist inside the vacuole. This high pressure would facilitate absorption of the gas by any liquid which was able to penetrate the membrane. On the other hand, this conclusion can be criticized on the grounds that Czapek's values for the surface tension of protoplasm are not generally accepted, and that it is doubtful whether the conception of turgor pressure can legitimately be applied to blue-green algae in which vacuoles containing sap are rare and in which the cell is not surrounded by a wall of the type usually met with in plants. Moreover, in considering the magnitude of the surface tension arising at the surface of the protoplasm, Klebahn uses the value for the surface tension at an air-water interface although the algal cells would be in contact with water (see also p. 214).

Klebahn also attempted to determine the composition of the vacuole-gas. The slowness with which alkalies or alkaline pyrogallol effect absorption of the gas suggests that neither carbon dioxide nor oxygen can be present in any quantity. It must be remembered, however, that the speed of absorption depends rather on the effect of the reagent on the vacuole membrane than on its efficiency as a gas-absorbent. The results of analysis of the gas, obtained in the manner already outlined (p. 211), are without great significance, since the bulk of the gas is not derived from the vacuoles. Moreover, preserved material was used in these experiments. A larger proportion of nitrogen was always found than would be expected if the gas were dissolved air. No inflammable gases were obtained. So far as it goes, the work indicates that the gas is probably nitrogen, but the problem is far from settled. It is possible that the gas may differ from species to species. Klebahn suggests that the vacuole membrane may be permeable to oxygen, which, if present, would then be withdrawn to supply the needs of respiration.

(3) *Discussion of the gas-vacuole theory*

One objection to the gas-vacuole theory has been based on the argument that the forces opposing bubble formation in protoplasm must be extremely great and that, even where metabolism results in vigorous evolution of gas, as in yeast or the assimilatory tissues of higher plants, gas-vacuoles are never formed (Molisch, 1903). If the production of gas is distributed over a comparatively extensive region of the cell and if it is able to diffuse away rapidly when once formed, it is unlikely that the partial pressure will ever be such as to lead to the formation of bubbles. Evidently one or both of these conditions are not fulfilled in the Myxophyceae possessing gas-vacuoles. Either the production of gas is concentrated in certain centres or its free diffusion is impeded in some way. In the latter connexion it should be emphasized that the protoplasm of blue-green algae shows peculiar features and that it is probably more gel-like in consistency than that of other plants.

The gas-vacuoles are rarely spherical, but this cannot be taken as an objection to the theory, since gas bubbles enclosed in colloids may possess very irregular shapes. The fact that the protoplasm of the cell is unlikely to be homogeneous would be sufficient to account for the irregular shapes assumed by the vacuoles.

The arguments in favour of the view that gas-vacuoles are surrounded by a membrane have already been mentioned (p. 212). Klebahn's assumption that this membrane is impermeable to gases or to one particular gas, does not seem to be necessary; all that is required is that the membrane should be sufficiently rigid to prevent the collapse of the cavity and the entry of water. Once the membrane is formed, the gas, initially at a pressure depending on the size of the bubble and the other factors discussed on p. 212, can diffuse away until equilibrium is reached. In this connection it is of interest to note that for a given amount of alga the volume of gas-vacuoles and the volume of gas do not differ very widely, which suggests that the gas is approximately at atmospheric pressure. No matter what gas was initially present, if the membrane were permeable, the equilibrium composition would be determined by the requirements of the alga and the nature of the gases in the environment.

The nature of the vacuole membrane is obscure, although its ready destruction by traces of fat solvents suggests that it may be of a lipide nature. Possibly it consists of a single layer of orientated molecules. The reddish colour usually displayed by gas-vacuoles was ascribed by Klebahn (1929) to chromatic aberration of the microscope lens but this is very small in modern instruments and usually tends towards the blue (cf. van Goor, 1925). The latter points out that the colour effect cannot be an optical illusion, produced as a colour complementary to the blue of the surrounding cell material, since it persists in decolorized material. He also maintained that it could not be due to interference, since this would vary with the size and shape of the gas-vacuole. The persistence of the colour after treatment with various reagents renders it unlikely that it is caused by a pigment. There is a possibility, however, that it may be an interference effect, due not to the gas, but to the membrane, a suggestion which lends support to the idea that the latter is a monomolecular layer. It must be emphasized that the properties of gas-vacuoles may not depend so much on the gas as on the membrane which encloses it.

V. THE FUNCTION AND ECOLOGICAL SIGNIFICANCE OF GAS-VACUOLES

(1) *Historical review*

Richter (1894) held that the structures in question played some important part in metabolism, as do the granules of rather similar appearance in *Beggiotoa*. Later workers (Strodtmann, 1895; Klebahn, 1895; Molisch, 1903) thought that they were produced specially to enable the alga to float, a view opposed by Brand (1901) and Fischer (1905) (see p. 207). Lemmermann (1910) suggested that the vacuoles might constitute a screen against strong light, a view supported by insufficient evidence. Lauterborn (1915), who considered that increase of the floating capacity could scarcely be the chief function of the "pseudo-vacuoles", concluded that they were specially characteristic of species inhabiting the sapropel (mud lacking oxygen and containing soluble organic matter, carbon dioxide, methane and hydrogen sulphide) and that they represented a labile metabolic product which enabled the alga to survive periods of oxygen shortage by acting as a source of energy. The chemical nature of the substance involved was not suggested, however, nor was any mechanism indicated whereby it could act as a source of energy. Later Kolkwitz (1928), reporting on the presence of gas-vacuoles in the bacterium *Sarcina ventriculi* from the bottom of a stagnant pond, suggested that the forms possessing these structures were mud-inhabiting types and that the gas in the vacuoles was produced by fermentation under anaerobic conditions. This idea was further developed by Canabaeus (1929) who subjected species of *Anabaena, Tolypothrix, Calothrix, Phormidium* and *Oscillatoria* to anaerobic conditions, using for this purpose a stream of hydrogen; no details are given as to the method of purification, if any. In other experiments she exposed the alga to certain concentrations of sodium chloride, of ferric sulphate or of sodium chloride and potassium monohydrogen phosphate. By either method she was able to induce the formation of gas-vacuoles in species in which they are not normally found, although this occurred only under definitely circumscribed conditions called the gas-vacuole level. The opinion is expressed that limitations of technique alone prevent this level being found for all blue-green algae. Since gas-vacuoles frequently appear in the vicinity of the phycocyanin granules, which are nitrogenous in nature, the latter are regarded as the substrate by the fermentation of which the gas is produced. This fits in with the suggestion that this gas is nitrogen. Canabaeus claims that an enzyme which is supposed to be stored in the heterocysts, is responsible for the fermentation, but the evidence on this point is entirely insufficient.

(2) *Discussion*

Forms producing gas-vacuoles are therefore regarded as typical of sapropelic or other habitats where the "gas-vacuole level" of the species concerned is reached. It is a fact that many Myxophyceae inhabiting sapropel and bottom muds—habitats that are characterized by oxygen shortage—contain numerous gas-vacuoles (Utermöhl, 1925, p. 294). Bethge (1935) records the appearance of a planktonic species of *Chroococcus* with gas-vacuoles at a time when the water contained hydrogen sulphide and the only other organism present was the sulphur bacterium *Chromatium okenii* so that acute oxygen shortage might be expected. The planktonic algae possessing gas-vacuoles are regarded as developing in the bottom mud and rising to the surface of the water when the conditions are such that gas-vacuoles are formed. Such a view, which would account for the sudden appearance of an abundance of water bloom, has frequently been expressed (Wesenberg-Lund, 1904;

van Goor, 1925; Canabaeus, 1929; Bethge, 1935), but, apart from an observation of Wislouch (1912) who recorded the rising of a bottom skin of planktonic algae to the surface, the presence of the species involved in a living condition in the bottom mud has never been demonstrated. The spores of species forming water blooms are without gas-vacuoles (Klebahn, 1895) and are heavier than water.

Apart from the regular occurrence of gas-vacuoles in species producing water-blooms and in those inhabiting sapropel, they are also found in others. Thus *Nostoc linckia*, a soil-inhabiting species, shows gas-vacuoles during active germination, as well as during the formation of hormogones (Klebahn, 1922, p. 545). Unfortunately it is extremely difficult to obtain successful cultures of species containing gas-vacuoles, so that observations on the relation of the latter to stages in development and during cell division are scanty. According to Canabaeus (1929) material in which gas-vacuoles have been produced artificially, shows no further development and dies, unless the vacuoles again disappear. In Klebahn's (1929) cultures of a species of *Anabaena* with gas-vacuoles, no more of the latter were produced, although growth was vigorous.

Apart from the work of Canabaeus, there are no experimental data on the effect of external conditions upon gas-vacuole formation. Gas-vacuoles seem to originate as a result of some metabolic process in conjunction with a condition of the protoplasm which facilitates bubble formation, one or both of these factors appearing to depend on special environmental conditions. The problems are still largely unsolved and our knowledge of the diverse factors involved is still very meagre. The role of the vacuoles as a means of flotation seems to be merely incidental.

VI. SUMMARY

A critical account is given of the present state of knowledge of the gas-vacuoles which are characteristic of many blue-green algae. The distribution in nature and the properties of these vacuoles are described. The various theories of their nature are discussed, the available evidence indicating that they are spaces in the protoplasm containing gas. It is emphasized that the properties of gas-vacuoles may depend not so much on the gas as on the membrane which encloses it. A review of the various suggestions as to the origin and significance of these structures is given. It is probable that they are connected in some way with anaerobic conditions prevailing in the environment and that the capacity to float which they confer upon the alga is incidental. No satisfactory conclusion as to their significance has, however, yet been reached.

In conclusion I wish to express my thanks to Prof. F. E. Fritsch, F.R.S., and to Dr F. M. Haines for valuable suggestions and criticism in connection with the preparation of this review.

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THE STRUCTURE AND EVOLUTION OF THE HOLOSTEAN FISHES

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I. INTRODUCTION

Our knowledge of the structure and classification of the Holostei is due largely to the works of Smith Woodward, especially the *British Museum Catalogue of Fossil Fishes* (1889–1901); later this was supplemented by the monograph on Wealden and Purbeck fishes (1916–19). These and numerous shorter papers form the basis for all modern work. While dealing with the various Triassic faunas Brough (1939) was brought into contact with some of the earlier Holostei and has described members of the Eugnathidae and the relation of that family to the Amiidae. Brough also contributed an article on the evolution of Triassic bony fishes to *Biological Reviews* in 1936, to which the present review is in some measure a continuation. I have examined representative genera of six of the holostean families, considering especially the structure of the neurocranium and its ossifications, and the relations of these to the history of the order.[1]

The four major groups of the Actinopterygii, which have recently been conveniently defined by Brough (1939), have been dominant during successive geological periods:

Palaeoniscoidea: Carboniferous and Permian
Sub-Holostei: Trias
Holostei: Jurassic and Lower Cretaceous
Teleostei: Upper Cretaceous and onwards

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[1] This research on neurocrania was completed before the present article, but it has not yet been possible to publish it. Consequently several statements on neurocranial structure appear here for the first time, and in a short general review there is no space to give the detailed evidence on which they rest. Figs. 2, 3 A, 10 and 12 were intended also to be copies of those in the earlier work.

Although these groups can be separated by the exact structural definitions necessary for systematic divisions, it is also true that they do to some extent merge into one another and that there is a general structural progression throughout the bony fishes.

According to Brough (1939, p. 109) the Holostei may be diagnosed thus:

'Maxilla and preoperculum reduced and completely separated and the preoperculum narrow dorsally. The tail is hemiheterocercal, or rarely, homocercal. Dorsal fin rays few and equal endoskeletal supports in number.'

The earliest known holostean is *Acentrophorus* (Semionotidae) from the Upper Permian Marl Slate (Gill, 1923); the published lists of the Triassic faunas contain various genera of the Eugnathidae, Semionotidae, Macrosemiidae and Pholidophoridae (see Brough, 1931), but some of the older identifications were made before the vast variety of the sub-holosteans was realized and they may not be entirely reliable; this applies particularly to the last-named family. Nevertheless, it seems to be fairly certain that the holosteans multiplied rapidly in extreme upper Triassic times, and that the faunas of the period do contain some characteristic eugnathids, semionotids and pholidophorids; moreover, these families appear to have been as distinct at that time from one another, and from the sub-holosteans, as they were at later stages in their history.

The most important character separating the Holostei from the sub-Holostei concerns the preopercular, maxilla, and jaw suspension; in the earlier order the preopercular is normally broad and plate-like, or occasionally palaeoniscoid (i.e. in two limbs, the upper expanded and firmly fixed in the cheek structure), whereas in the holosteans it has become reduced dorsally and is usually thin and crescentic. This transition is only a further stage in the morphological sequence that began in the palaeoniscoids with the breakdown of the rigid structure of the cheek, a decrease in the backward thrust of the jaw suspension (connected with long jaws and predacious habits), and a freeing of the maxilla and preopercular. Some of the Holostei have long, predacious jaws and a backwardly swung articulation, the hyomandibular lying obliquely as a result, but in such cases the mechanism is quite different from the palaeoniscoid.

The body skeleton in the sub-Holostei and Holostei is very similar, the diagnostic reduction of the endoskeletal fin supports in the latter being a small difference. The sub-holostean neurocranium has so far always been found as a solid structure of one piece, though presumably it arose from separate ossifications in ontogeny; the degree of ossification varies rather haphazardly among the adult Holostei, but more often than not the bones are separate, and there seems to be a characteristic bone pattern varying only in detail between family and family.

II. STRUCTURE AND CLASSIFICATION

The holosteans have a number of characters which, while they cannot be included in an exact diagnosis because they may not apply to some of the most aberrant members, yet serve to make the 'typical' fishes easily recognizable. In nearly all the fossil forms the tail is hemiheterocereal, with only a short upper scaly lobe. Fulcra are usually present on all fins, and the pectoral fin is inserted into a very short scaly lobe, the anterior rays being slightly the higher; although distinctly flexible its mean position was probably horizontal.[1] The pattern of the roofing bones of the skull is very stable (shown in Fig. 2); there is a single small rostral with the ethmoid commissure of the sensory canal system, a pair of nasals loosely attached to the rest of the skull, long frontals and rather short square parietals; dermopterotics longer than the parietals and small dermosphenotics bearing the right-angle bend of the infraorbital sensory canal behind the eye. The variation in the sensory canals on the posterior roofing bones, especially the course of the supraorbital canal which may continue on to the parietal or may fuse with the infraorbital on or near the dermosphenotic, has been thought on the evidence of the development of *Amia* to be of no great significance.

The palate has a very similar structure from the earliest genus, *Acentrophorus*, (Gill, 1923, p. 32) to the most advanced, *Leptolepis* (Fig. 11). Presumably, once the holostean method of jaw suspension was instituted, the palatal structure was largely determined by mechanical necessities, and there was little scope for change. The lower jaw of most Holostei consists of dentary, angulo-articular, surangular and prearticular elements, and the relations between these bones does not vary much; the important exception is group V described below, in which they are considerably reduced in number. One of the clearest links between the Holostei and sub-Holostei is that of mandibular structure, for one of the most advanced families of the latter, the Ospiidae, has a typical holostean lower jaw. Lastly, the neurocranial pattern is known in eight holostean genera and shows considerable similarity in both shape and position of the bones (Fig. 12), with some differences in the posterior otic region and an occasional development of a supraoccipital.

From the occasional divergences from these 'typical' holostean structures, and from consideration of those parts of the skull and body skeleton which are less constant in structure, it is possible to divide the families of the Holostei into five groups.

GROUP I. EUGNATHIDAE, AMIIDAE, MACROSEMIIDAE

The eugnathids are one of the least specialized of the holostean families; they have rather elongated bodies and well-developed jaws with sharp teeth. The best known genera are *Eugnathus* (? Upper Trias—Kimmeridgian), *Heterolepidotus* and *Caturus* (? Upper Trias—Lower Cretaceous), and these provide a fairly full account of the osteology of the family. The scales are very rarely deepened on the flank, the tail is deeply forked and the fulcra conspicuous. The skull structure is best described by drawings (Fig. 2), but particularly characteristic are the very small parietals and long frontals; the supraorbitals are normally few in number and dermosphenotic fairly large, the tendency to split into ossicles being a generic character of *Caturus*. The cheek-plate pattern is characteristic of the family, and even more important are the maxilla and supramaxilla; the former varying in length with the jaws, but characteristically with a slight nick in the hind border, the latter flat below and rounded above. The lower jaw consists of a long shaft-like dentary forming the anterior part, rising rather suddenly to the coronoid process behind, which carries the surangular; the angular and articular are normally fused and the inside of the jaw bears a prearticular with numerous small teeth.

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[1] Many restorations of the pectoral fin (including some reproduced in this article) show it sloping downwards from behind the cleithrum, the anterior rays set lowest on the flank. This position in the fossils is probably due to lateral crushing. This point is noted by White (1939) in a review of the actinopterygian pectoral fins.

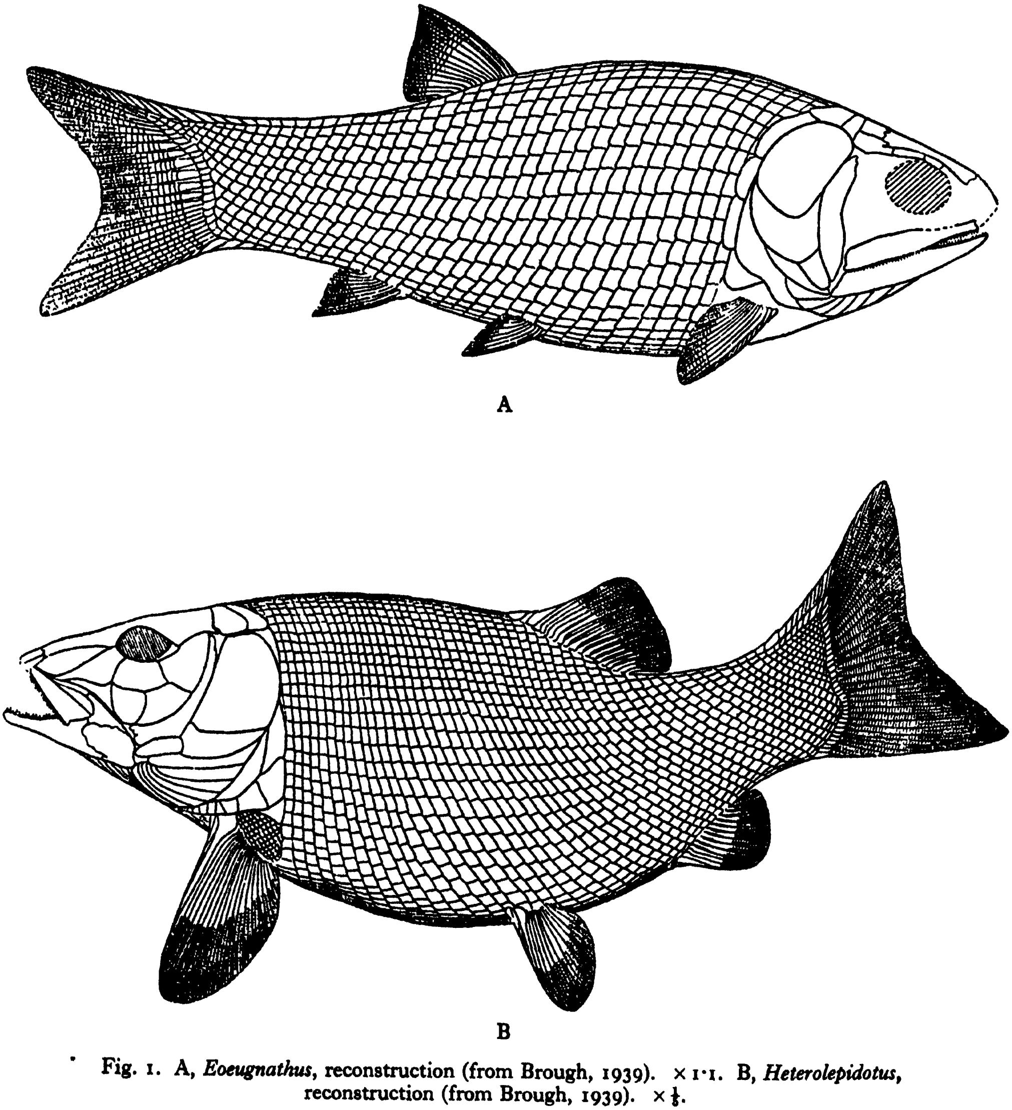

Fig. 1. A, *Eoeugnathus*, reconstruction (from Brough, 1939). × 1·1. B, *Heterolepidotus*, reconstruction (from Brough, 1939). × ½.

The neurocranium is best known from *Caturus* but is supplemented by imperfect specimens of several other genera—*Eurycormus* (Smith Woodward, 1890), *Osteorachis* (Smith Woodward, 1897) and *Heterolepidotus* (Brough, 1939). It was well though

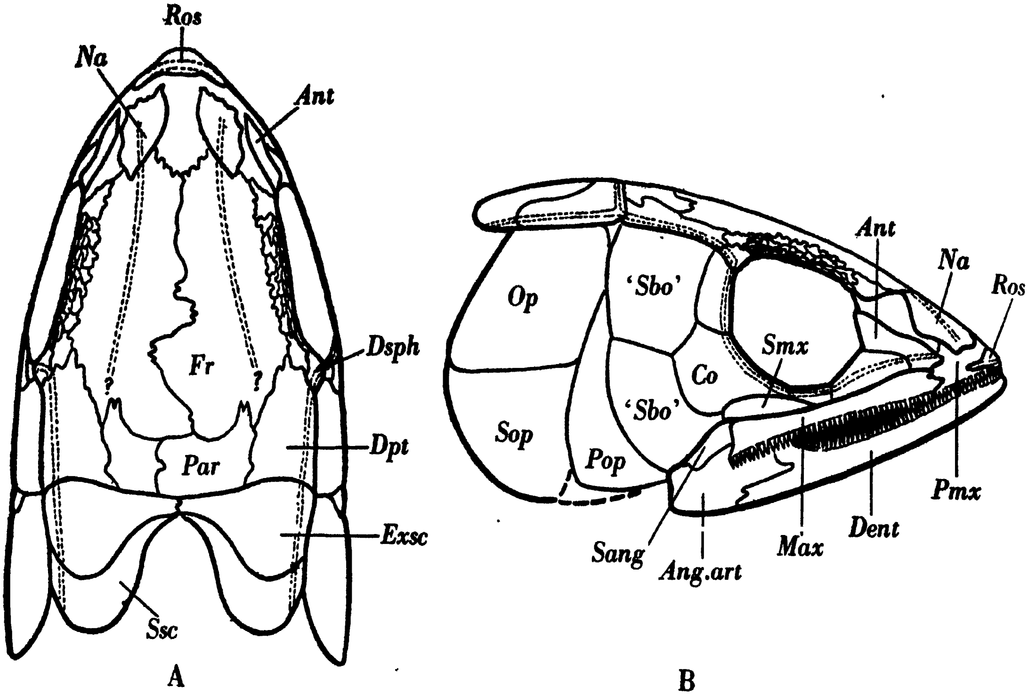

Fig. 2. *Caturus*, reconstruction of the dermal bones of the head. A, dorsal view. B, side view. × ⅓. *Ang.art*, angulo-articular; *Ant*, antorbital; *Co*, circumorbital; *Dent*, dentary; *Dpt*, dermopterotic; *Dsph*, dermosphenotic; *Exsc*, extrascapular; *Fr*, frontal; *Max*, maxilla; *Na*, nasal; *Op*, opercular; *Par*, parietal; *Pmx*, premaxilla; *Pop*, preopercular; *Ros*, rostral; *Sang*, surangular; '*Sbo*', 'suborbital'; *Smx*, supramaxilla; *Sop*, subopercular; *Ssc*, suprascapular.

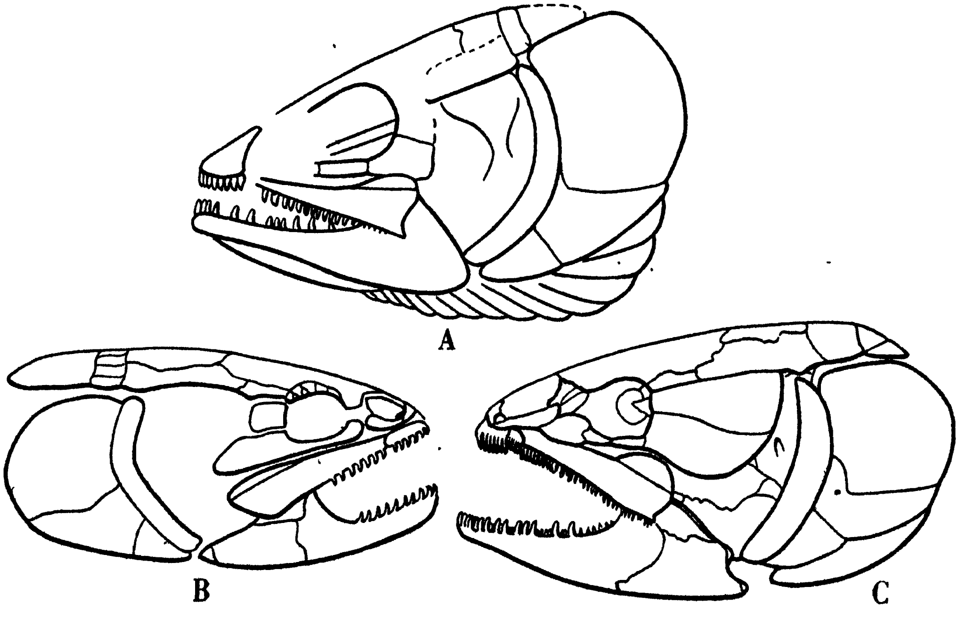

Fig. 3. Some amiid skulls in side view. A, *Megalurus*. × 2. *B*, *Sinamia* (from Stensiö, 1935). × 1·5 approx. C, *Amia* (from Allis, 1897). × 1·25.

not completely ossified; the hyomandibular facet had a facing of cartilage, and there is a considerable gap in the ossification in the centre of the roof of the postorbital section and of the ethmoid region. Although the orbit is large there is an almost complete bony septum between the eyes, with only a small interorbital fenestra immediately in front of the optic foramen. The bones of the brain case were probably partially fused in the adult, and the only cartilage bones known at all certainly are the autosphenotic, epiotic and those of the occiput. The intercalar in all holosteans is a superficial membrane bone with a ligamental attachment to the suprascapular; it varies more than the rest, and in the eugnathids is large, with a large process and conspicuous band of fibres on the lateral wall. There is no supraoccipital and it is not known whether there was a separate 'ganoid' opisthotic in the posterior otic region or not.

There is little important divergence in cranial structure among these genera already mentioned. *Eugnathus* is longest and slimmest; longest also in the jaw and frontals with a distinctly backwardly thrust suspensorium. *Heterolepidotus* is stouter with a much shorter jaw and suspensorium inclined slightly forwards; the orbit is rather small and as a result the circumorbital series below the eye is unusually stout. *Caturus* comes between the other two in proportions of body and mandible, has the numerous supraorbital ossicles, and scales small and rather thin. The earliest established eugnathid is *Eoeugnathus* from the Middle Trias of Besano (Brough, 1939, p. 71); it is probably more closely related to *Heterolepidotus* than to any other genus. It is not very elongated in the body, but has a typically eugnathid pattern of roofing bones. The opercular is rounded, the maxilla has the beginnings of a eugnathid shape, the supramaxilla is unfortunately not known. It is the mandible that is most strikingly primitive; a coronoid process is present but the sharp difference between anterior shaft and posterior coronoid region has not yet been developed.

There are other eugnathid genera less completely described and mostly more specialized and restricted. *Osteorhachis* is a large form resembling *Caturus* from the Lias and Oxfordian. *Eurycormus* (Oxfordian and Kimmeridgian) is a thin-scaled type; *Neorhombolepis* (Cretaceous) has vertebra completely ossified in the form of discs.

The Amiidae include the genera *Liodesmus*, *Megalurus*, *Sinamia* and the living *Amia*. *Megalurus* (Kimmeridgian and Purbeck) shows points in common with both *Caturus* and *Amia* and suggests strongly a eugnathid ancestry for the latter. This was also deduced independently by Brough from a study of *Heterolepidotus latus*. *Megalurus* has a eugnathoid type of skull and pectoral fin, though rather short in the jaw and small in the orbit, but the rest of the body is amioid. The caudal fin has a convex posterior border and externally is almost symmetrical above and below. The abdominal hypo- and pleurocentra have fused to form solid constricted cylinders while those of the caudal region remain separate discs; the dorsal fin is somewhat elongated. Thus *Megalurus* is at a halfway stage in the structural changes that have brought about the evolution of *Amia*, changes which can be summed up as follows: great degeneration in the neurocranium which is largely cartilaginous; decrease in the size of eye, seen in the dermal bones and especially in the flattened neurocranium with a robust interorbital septum; scales thin and cycloid; elongation of the dorsal fin; development of a homocercal tail; dorsal migration of the pectoral fin, which is small and rounded and situated rather high on the flank. The head bones show their eugnathid inheritance particularly in the shape of the maxilla and supramaxilla and mandible; also bones of the snout decidedly resemble those of *Caturus*. On the other hand the parietals are normally fused into one large central bone quite half the length of the frontals, and two large cheek bones supplant the double series of circumorbitals and 'suborbitals' found behind the eye in nearly all fossil ganoids.

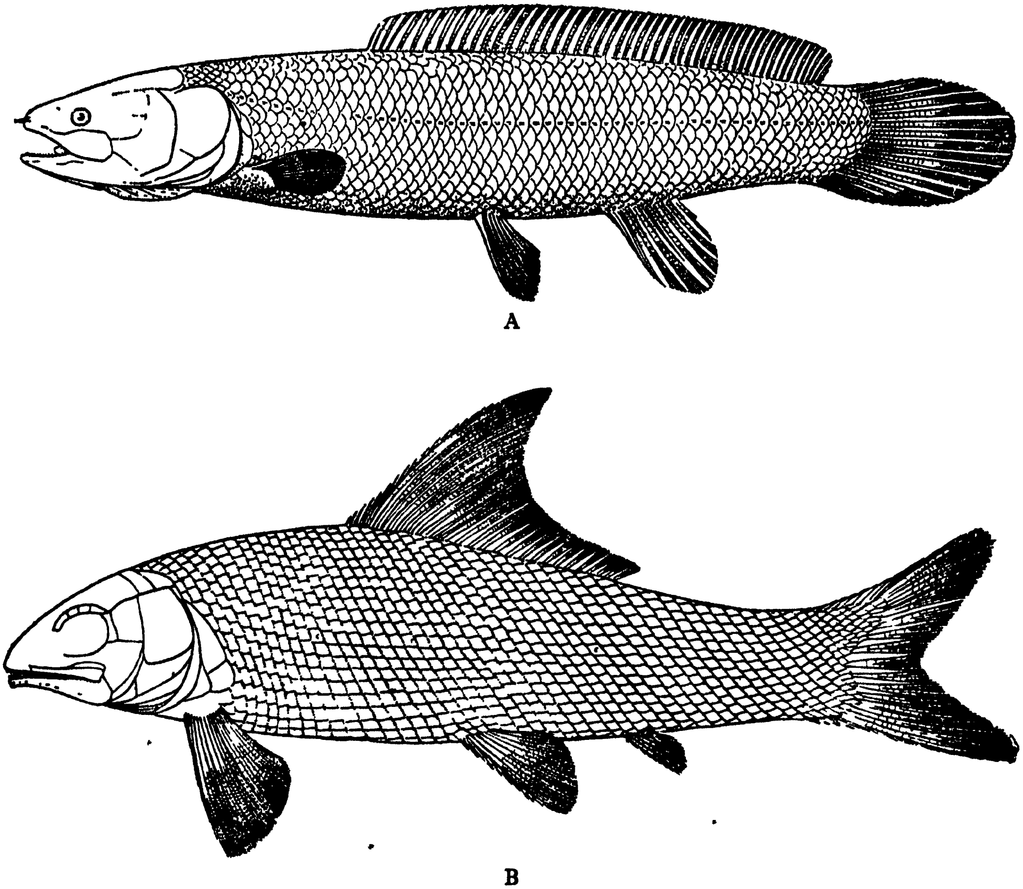

Fig. 4. A, *Amia*, reconstruction (from Goodrich, 1909). B, *Ophiopsis*, reconstruction (from Smith Woodward, 1918). × $\frac{2}{3}$ approx.

Sinamia is a form found in the Lower Cretaceous of China (Stensiö, 1935); it is clearly closely related to *Amia*, and is scarcely distinguishable in neurocranium and dermal roofing bones. The uppermost circumorbitals have not developed the large backward extension they have in *Amia*, and the dermal bones and the rhombic scales retain a thin layer of ganoine. The row of small extrascapulars, slight difference in shape of maxilla and small supraorbital ossicles remove *Sinamia* but slightly from the direct ancestral line.

The Macrosemiidae are connected with the Eugnathidae through their earliest genus *Ophiopsis* (? Upper Trias—Lower Cretaceous), many of the dermal bones of which are almost indistinguishable from those of that family, especially maxilla, supramaxilla, cheek plates and circumorbitals. The parietals, however, are larger, and the frontal has a very characteristic lateral embayment accommodating three supraorbital bones and expands again in front of these; most easily recognized of all is the shape of the lower jaw, the coronoid expansion being not well marked and the lower margin decidedly concave in the anterior region, as a result of which *Ophiopsis* has a peculiar underhung appearance. The neurocranium is unfortunately not known. The scales are small and scarcely, if at all, deepened on the flank, and frequently serrated at the hind border; the genus also has in a moderate degree the elongation of the dorsal fin which characterizes the family. The notochord ossifications are small, delicate, pleuro- and hypocentra.

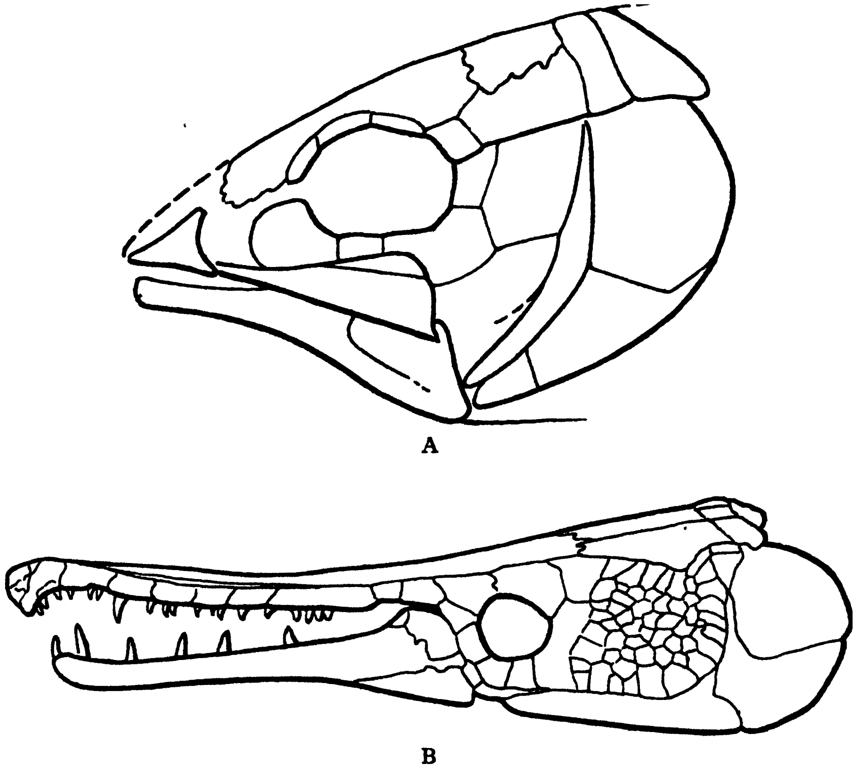

Fig. 5. A, *Ophiopsis*, reconstruction of the dermal bones in side view. × 2·25. B, *Lepidosteus* dermal bones in side view (after Smith Woodward, 1895).

The remaining genera are very little known in the head region, but from this eugnathoid-like beginning they appear to have developed into somewhat elongate delicate fishes, rather small in the mouth; they show an increasing elongation of the dorsal fin (divided into two in *Propterus* and *Notagogus*) and a tendency to degeneration in the squamation, e.g. *Macrosemius*. The latest representative is *Petalopteryx* (Upper Cretaceous) with irregular scales and greatly divided cheek plates.

GROUP II. SEMIONOTIDAE, LEPIDOSTEIDAE

The Semionotidae is an ancient family among the Holostei, for the Upper Permian *Acentrophorus* is a typical, if slightly primitive, member; *Semionotus* appears frequently in Upper Triassic faunas and *Lepidotus* and *Dapedius* appear in the Lower Lias. The family is characterized by a grinding dentition of numerous rounded or peg-like teeth; the head bones and scales are massive, the latter undeepened, and the fulcra particularly stout and conspicuous; except in the much deepened *Dapedius* there is a slight excavation at the base of the dorsal fin. Even including this genus the dermal bones vary very little; the mouth is short and the maxilla short and rounded with a small supramaxilla perched on top of it, and there is a large number of small square circumorbitals which vary slightly in number from specimen to specimen.

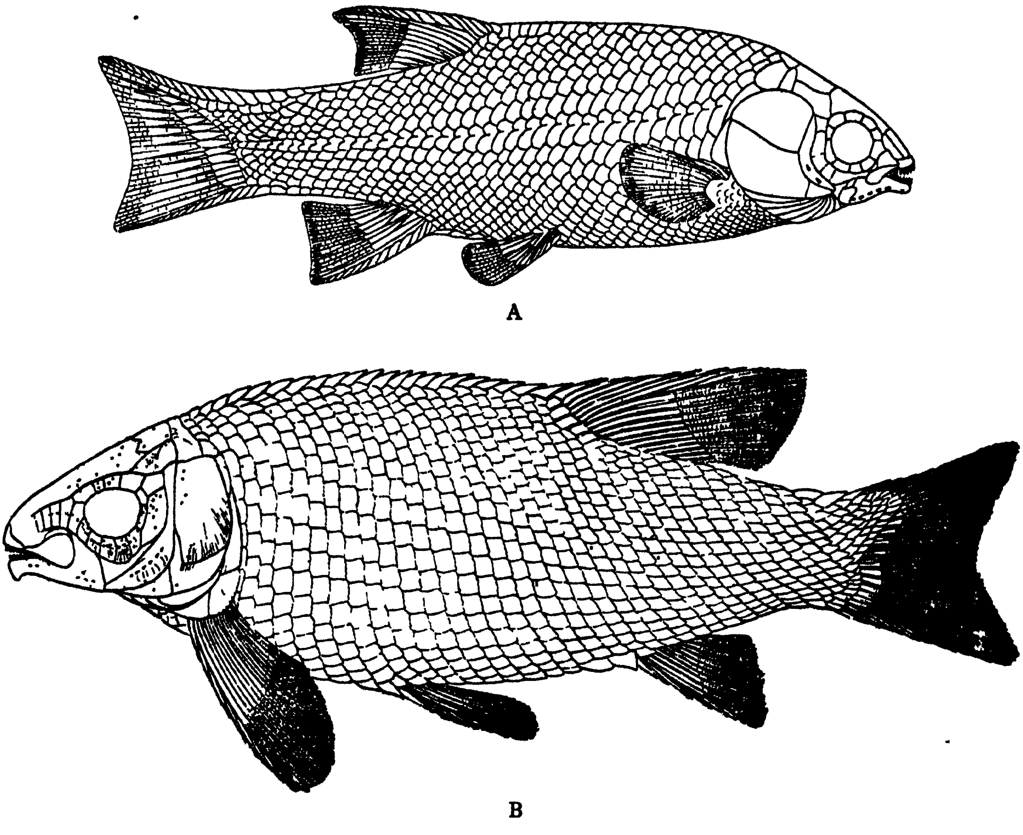

Fig. 6. A, *Acentrophorus*, reconstruction (after Gill, 1923). × 1·5 approx.
B, *Lepidotus*, reconstruction (from Smith Woodward, 1919). × ½ to ⅓.

Acentrophorus (Gill, 1923) subscribes to most of the typical semionotid characters except that the scales are rather thin and the teeth conical rather than grinding. Moreover, the pectoral fin has a peculiarly modern appearance in that it is small and situated rather high on the flank; all the fulcra are very massive. The tail fin is frankly primitive, the scales of the upper lobe still extending to the extreme tip though in the hind half there is only a single row of them. This and the unspecialized teeth suggest that *Acentrophorus* is very near the base of the semionotids.

The neurocranium has been described in both *Lepidotus* and *Dapedius*. It is very fully ossified in the latter, to the obliteration of most of the sutures; in addition it is rather unusual in shape owing to the deepening of the whole head and the back is much excavated for the insertion of trunk muscles. *Lepidotus* is much more normal in shape and has a fair proportion of cartilage in the neurocranium, especially in the posterior otic region, a ganoid 'opisthotic' being quite definitely missing, as is also the supraoccipital; the intercalar is small and star-shaped. The ethmoid region is little known but there was some bone around the olfactory capsules.

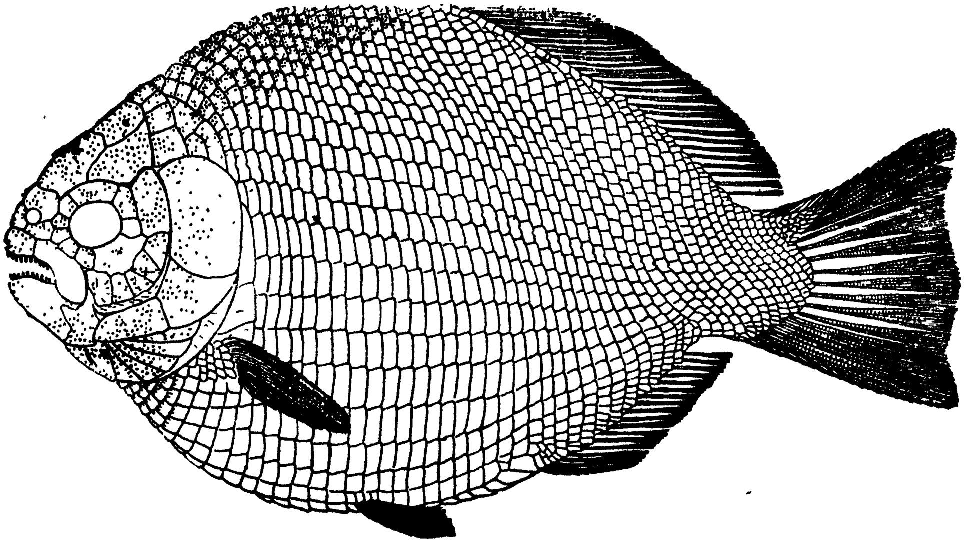

Fig. 7. *Dapedius*, reconstruction (from Smith Woodward, 1895).

The inclusion of the Lepidosteidae in group II depends largely on the similarity of the neurocranial ossifications in the living *Lepidosteus*—the sole genus—to those of *Lepidotus*. The same cartilage bones are present and in roughly the same positions, allowing for the flattening of the skull in the recent genus, the greater amount of cartilage and some reduction in the size of the eye; the intercalar is lost, probably owing to the development of a different attachment of the shoulder girdle; the basipterygoid process is unusually large, possibly in response to the development of a rostrum, and has involved the prootic as well as the parasphenoid. The rostrum has altered the dermal bones very much, so that the homologies of some of the anterior ones is controversial, but the chain of square circumorbitals remains, behind which is a mosaic of small irregular plates. The body of *Lepidosteus* is much elongated like the head, with dorsal and anal fins placed far back and caudal fin rounded posteriorly; the scales are ganoid and rhombic and the vertebrae complete rings of fused pleuro- and hypocentra.

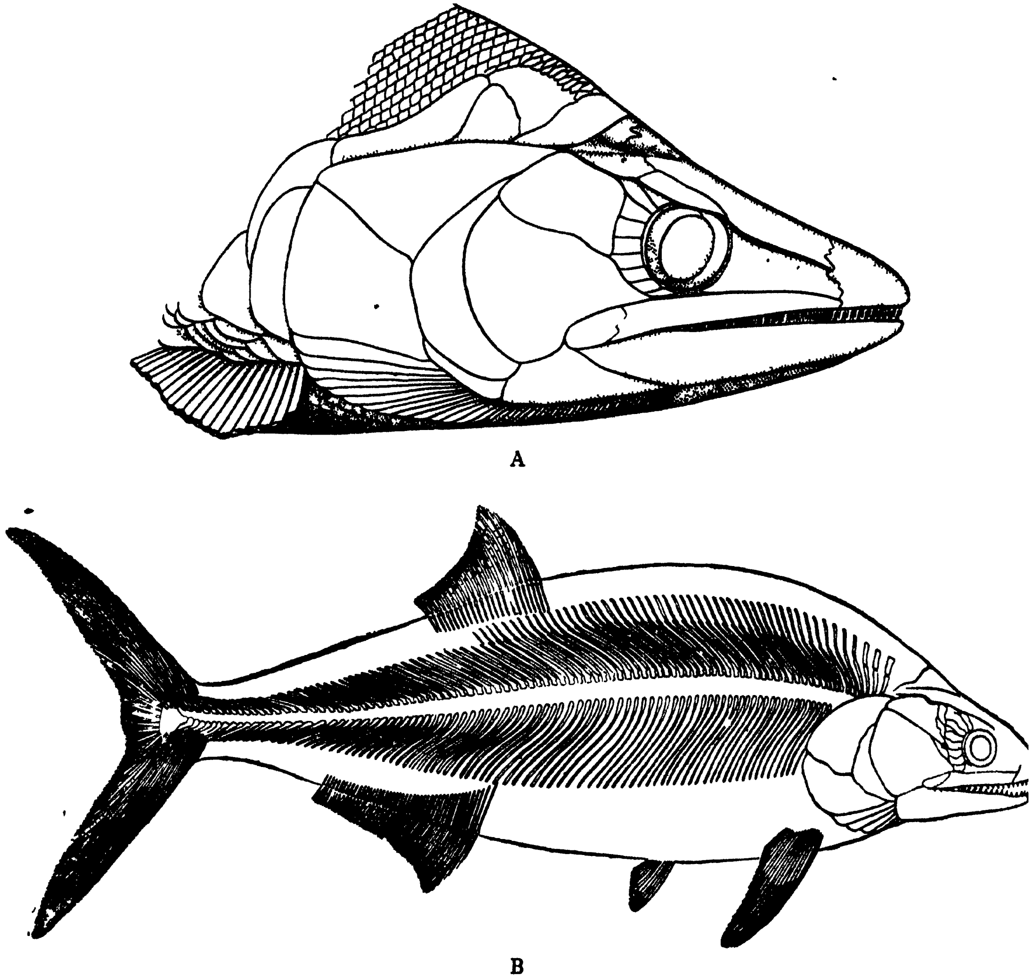

Fig. 8. A, *Pachycormus*, head in side view (from Smith Woodward, 1908). B, *Hypsocormus*, reconstruction (from Smith Woodward, 1895).

GROUP III. PACHYCORMIDAE

This group is a small and comparatively late one, known from the Upper Lias, and is increasingly specialized to a 'sword-fish' type of habit; the two best known genera, *Pachycormus* (Upper Lias) and *Hypsocormus* (Middle and Upper Jurassic) have been shortly described from this point of view by Smith Woodward (1908). The outstanding characteristic is the development of a rostrum; in *Pachycormus* this is quite short and rounded and is accompanied by a rounded elevation in the parietal region, apparently unsupported by any neurocranial structure. The remainder of the bones are of fairly normal holostean pattern without affinities to any other family in particular. The jaws are long with sharp teeth and suspensorium strongly thrust backwards, and the 'suborbital' plates in consequence are large. The body is rather deep, scales very small and tail strongly forked and powerful. *Pachycormus* has apparently lost the pelvic fins, a slight advance in specialization on other Liassic genera, *Sauropis* and *Euthynotus*.

Hypsocormus is very like *Pachycormus* for the most part, but the rostrum is long and the dorsal eminence lies largely behind the skull and the parietal region is therefore less prominent. The neurocranium is partially known in both genera and they have a very similar bone pattern. It includes a ganoid 'opisthotic' set in the side wall in front of the small intercalar; this bone is probably not found in any other holostean family and since the neurocranial pattern is remarkably stable this in itself would suggest the Pachycormidae to be an isolated group, and agrees with the points noticed in the dermal bones. The postorbital section of the neurocranium is otherwise well ossified but the bones are always distinct; in the preorbital section there are separate lateral and pre-ethmoid bones and probably a good deal of cartilage as well.

Protosphyraena (Chalk) is the most advanced genus, chiefly known from isolated rostra and some of the anterior head bones; the former is very long and must have been an effective weapon. Smith Woodward (1908) has suggested that with the increase in the length of rostrum the dorsal eminence would have moved farther and farther back, as it does in the sword-fishes, and that in *Protosphyraena* it may have been entirely behind the skull.

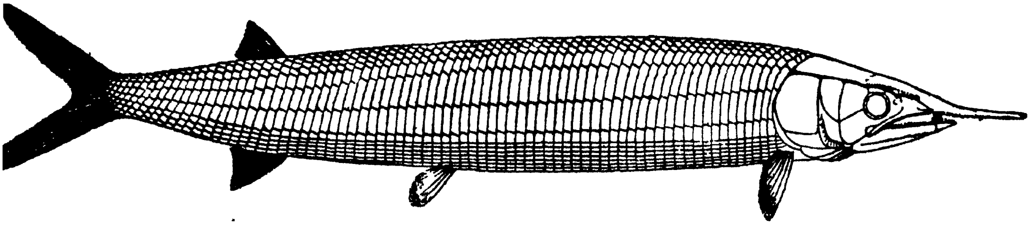

Fig. 9. *Aspidorhynchus*, reconstruction from (Smith Woodward, 1895).

GROUP IV. ASPIDORHYNCHIDAE

This is also an isolated group, ranging from the Middle Jurassic to the Upper Cretaceous; two closely allied genera are included, *Aspidorhynchus* and *Belonostomus*, both highly specialized with a long rostrum, elongate body and deepened flank scales. In the former the rostrum is prolonged far beyond the mandibular symphysis, in the latter scarcely at all. There is a superficial resemblance in shape and fused vertebral elements to *Lepidosteus*, but the cranial bones are entirely different.

The skull for the most part conforms to the holostean pattern with a few significant variations. There is only one series between the eye and preopercular instead of the normal two; the dermosphenotic is not present (or not in its usual form) in some species at least of *Aspidorhynchus*. The distribution of the sensory canals suggests that it may be fused to the frontal, or the right-angle turn of the infraorbital canal may have been transferred to the frontal and the peculiar triangular bone immediately below this may be the dermosphenotic. The neurocranium is less unusual, but is characterized by a small supraoccipital, seen on the dorsal surface only between the epiotics and parietals, and there is the large backward expansion of the prootic, possibly representing fusion with another posterior otic ossification, though there is no direct evidence of this. The neurocranium is particularly well preserved in some specimens from the Bathonian; it is rather high with a large interorbital fenestra; the bones of the postorbital sections have well-defined sutures but the preorbital is a solid piece. *Aspidorhynchus* is unusual among the Holostei in lacking a basipterygoid process.

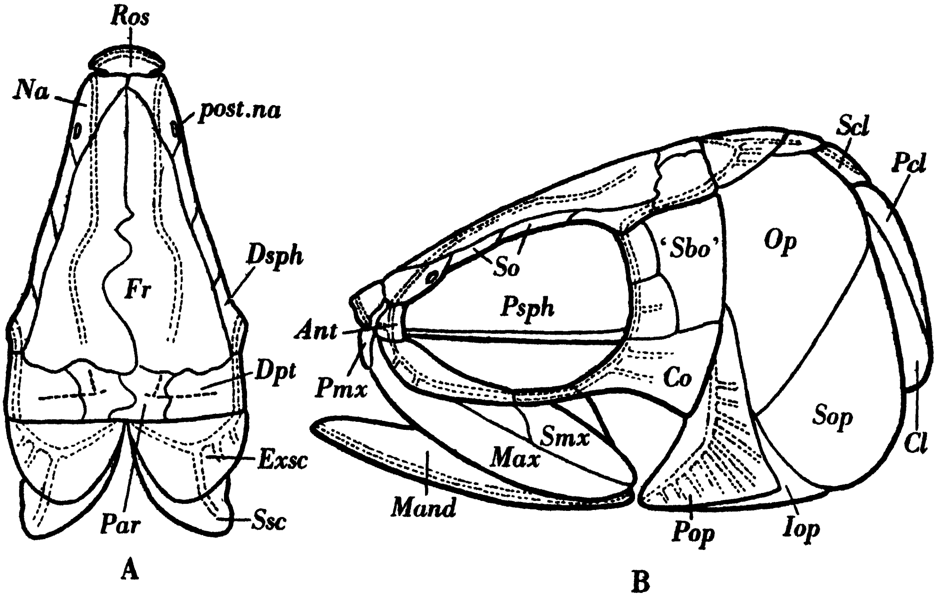

Fig. 10. *Pholidophorus*, reconstruction of the dermal bones of the head. A, dorsal view. B, side view. ×2. *Ant*, antorbital; *Cl*, cleithrum; *Co*, circumorbital; *Dpt*, dermopterotic; *Dsph*, dermosphenotic; *Exsc*, extrascapular; *Fr*, frontal; *Iop*, interopercular; *Mand*, mandible; *Max*, maxilla; *Na*, nasal; *Op*, opercular; *Par*, parietal; *Pcl*, postcleithrum; *Pmx*, premaxilla; *Pop*, preopercular; *post.na*, posterior nasal aperture; *Psph*, parasphenoid; *Ros*, rostral; '*Sbo*', 'suborbital'; *Scl*, supracleithrum; *Smx*, supramaxilla; *So*, supraorbital; *Sop*, subopercular; *Ssc*, suprascapular.

GROUP V. PHOLIDOPHORIDAE, LEPTOLEPIDAE, OLIGOPLEURIDAE

This is a group of considerable antiquity and the only one to give rise to any great number or variety of descendants. An undoubted pholidophorid occurs in the Middle Trias; *Leptolepis* was derived from *Pholidophorus* in the Lias (probably Upper Lias) and gave rise to the basal teleostean stock which is known from the Upper Cretaceous.

The roofing bones, snout, cheek and opercular apparatus of *Pholidophorus* have a typical holostean appearance, not very unlike those of the eugnathids, but the structure of the jaws and neurocranium show the group to be considerably removed from any other. It is the only one to have two supramaxillary plates, overlying a broad curved maxilla; the lower jaw has a pronounced coronoid process on the dentary, there is no surangular, and the teeth are small and delicate. In the braincase there is a large supraoccipital covering a considerable part of the hind surface; the semicircular canals are placed high up and the posterior and external canals jut out prominently. The latter is surrounded by a separate ossification, the autopterotic, which *might* from its position be homologous with the pachycormid 'opisthotic', except that the very great difference in general neurocanial structure makes the assumption rather sweeping; the different names are retained so that the matter may be left open. Below the labyrinth the neurocranium narrows rapidly and the sacculus shows up as a bulge in the basioccipital and prootic regions. The orbits are large and interorbital fenestra very large; the preorbital section is not well known, but was probably a single ossification.

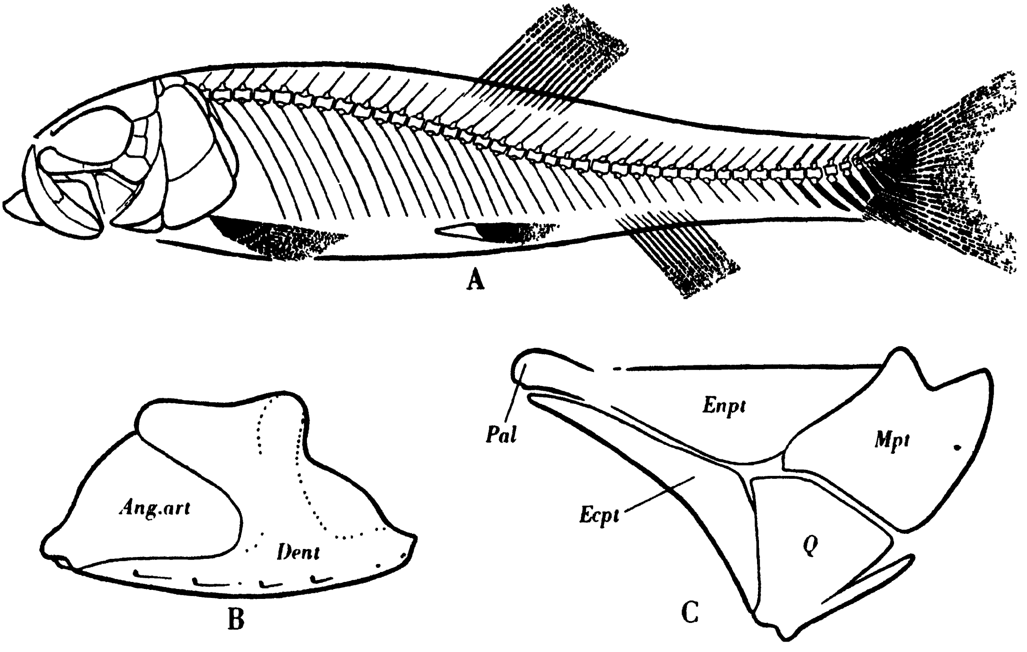

Fig. 11. *Leptolepis*. A, reconstruction, scales omitted. × 1·5. B, right lower jaw. × 10. C, left palate. × 6·6. (All from Rayner, 1937.) *Ang.art*, angulo-articular; *Dent*, dentary; *Ecpt*, ectopterygoid; *Enpt*, entopterygoid; *Mpt*, metapterygoid; *Q*, quadrate; *Pal*, palatine.

Many of the distinguishing features of *Pholidophorus* are passed on to *Leptolepis*, some becoming more exaggerated, together with some foreshadowing the teleosts. In particular the coronoid elevation is much more pronounced, situated unusually far forward on the jaw, which consists of dentary and angulo-articular only, the prearticular being lost. The teeth are reduced to very small outgrowths on the margin of the maxilla. The supraoccipital makes a small appearance on the skull roof for the first time, between the extrascapulars; in the teleosts it is to become an important element. The vertebrae, which in *Pholidophorus* are unfused pleuro- and hypocentra, have become fused into an hour-glass shaped centrum, though this still retains a small perforation for the notochord. The fulcra are reduced to a few minute scales on the upper caudal lobe. The external bones of the head have the usual layer of ganoine, though it is thin, but the cycloid scales are almost without it. In the transition to the earliest teleosts, the Elopidae, the two important changes are the loss of this ganoine entirely and of the perforation of the centra.

The Oligopleuridae (Upper Jurassic and Cretaceous) are a family of rather larger fishes probably slightly off the main *Pholidophorus-Leptolepis* line of evolution, but showing numerous advances parallel to the latter. The coronoid process is high and leptotepid-like; the vertebral elements fused and the dermal bones almost lacking in ganoine and scales thin. Fin fulcra, however, are still present.

The Holostei thus classified do not include the pycnodonts. Smith Woodward (1940) has recently reviewed the systematic position of this family and come to the conclusion that it is a late development of the sub-Holostei. Certainly the pycnodonts have few affinities with the rather conservative holosteans summarized above, particularly in their much expanded preopercular, fixed among the cheek plates; the parasphenoid also in *Mesturus* is short and palaeoniscoid, not extending to the back of the braincase as in the holosteans. Moreover, the group shows much divergence in the roofing bones and snout, including a large median 'supraoccipital' (Smith Woodward, 1895, Fig. 30). All these features are strongly alien to the Holostei and the removal of the pycnodonts from the order seems very desirable.

III. CONCLUSIONS

The variations in skeletal structure seen in the five groups can be analysed under various headings: there is the traditional holostean structure, the basis of the order; added to this there is the systematic variation of greater or lesser degree dividing it into groups, families and genera, as nearly 'natural' or phylogenetic as the very imperfect material will allow. Through these groups there is a certain amount of parallel evolution, producing not homeomorphs, the vertebrate skeleton being too complex for any far-reaching homeomorphy, but considerable resemblances on some points; lastly a moderate adaptive radiation is impressed on the fauna as a whole.

The most stable elements in the holostean skeleton have already been summarized; they are particularly skull roof, palate, and fin position and structure. The systematic variations may be assembled from the classificatory section; naturally they are concerned especially with those elements least likely to be affected by adaptation or subject to progressive (or parallel) evolution. The construction of maxilla and supramaxilla almost by itself will separate the order into the five groups, confirmed by the pattern of the cheek plates and of the neurocranial bones, if these are known; other features less universally useful are scale structure and deepening of the flank scales. Group V is also distinguished by the structure of the mandible.

First among the progressive tendencies is degeneration in ossification, which is exhibited in more than one way. The most striking is the replacement of bone by cartilage in the neurocrania of *Amia* and *Lepidosteus*, end points of two divergent lines of evolution. The *Pholidophorus*-teleost line does not apparently suffer much from this degeneration, at least among the holostean members, though it is interesting that in some living teleosts, e.g. *Salmo*, there is rather more cartilage present. The effect on the dermal bones is less sweeping, but the replacement of a few large bones of definite shape by a mosaic of small indefinite ones is usually regarded as bone degeneration; it most commonly affects the cheek plates and is found in *Lepidosteus* and the end point of the pachycormid line, *Protosphyraena*. The scales may also show degeneration in partial or complete loss of ganoine and in becoming cycloid. They are, for instance, thin in the eugnathid *Eurycormus* and have become cycloid in the Amiidae; *Macrosemius* also shows some degeneration, and in group V only the Pholidophoridae have the normal ganoid squamation, and even here there is possibly some reduction in the latest genera. The scales of *Leptolepis* are thin and cycloid and only have a very thin layer of ganoine, and those of the Oligopleuridae are similar; as already mentioned it is the complete loss of ganoine that provides one of the diagnostic characters of the Teleostei. With this reduction of the scales there is usually a corresponding reduction on the head bones.

The decrease in the size of the eyes seems to be confined to *Amia* and *Lepidosteus*. The elongation of the dorsal fin is found in both the Macrosemiidae and the Amiidae, in each case becoming more pronounced in the later members. The elongation in *Dapedius* is probably an unrelated phenomenon connected with the deepened shape of the body.

The last progressive type of variation is the fusion of the two halves of the vertebral centra into complete rings or solid cylinders; it affects several holostean lines: the Amiidae, *Neorhombolepis* among the eugnathids, *Lepidosteus*, the Leptolepidae and Oligopleuridae, and gives the body a deceptively modern appearance by being reminiscent of the teleosts.

There is a fair amount of adaptive radiation among the Holostei, though it is not so impressive as in the sub-Holostei; there are, for instance, no flying fish in the Jurassic faunas corresponding to the Triassic *Dollopterus*. The Eugnathidae are of the normal rather large carnivorous type, ranging up to a metre in length, with strong jaws and close-set sharp teeth; the Macrosemiidae were smaller, lighter and more elongated, but probably also fast-moving carnivores. The heavily built Semionotidae were clearly slower in habit; their grinding teeth are admirably suited to mollusc feeding, and they probably cruised at or near the bottom. The 'swordfish' type of adaptation found in the Pachycormidae has been already mentioned. The Aspidorhynchidae were elongated, quick-snapping fishes with very powerful swimming bodies; they vary considerably in size, but may be as much as a metre long. There remains the plankton-eaters—a place filled by shoals of small *Pholidophorus* and *Leptolepis*; the former has very small teeth and in the latter they are scarcely more than denticulations, and the jaw bones are thin and delicate also. These would take the place of the Triassic ptycholepids and the modern herrings.

Thus although the Holostei must have had a time of rapid evolution and divergence in the Upper Trias to produce the abundant Lower Liassic faunas, it did not take them to the extremes of diversity shown by their Triassic forerunners or Upper Cretaceous and later descendants.

So little is known of the early Triassic and Permian Holostei that the origins of the order are very obscure, and represent one of the greatest gaps in the history of bony fishes. The facts are simple: isolated members are known perfectly distinct before the Upper Trias, though occasionally slightly primitive in a generalized fashion. Beyond this almost everything is conjectural, including the difficult question of monophylogeny or polyphylogeny. The use of these words in itself raises the query—how small, relatively, must be the basal stock before a group is considered monophyletic? Among the holosteans the units concerned are fairly comprehensive and usually the family is considered in discussions on phylogeny. There is one family which can be traced backwards into the sub-Holostei, the Eugnathidae. Brough (1939, p. 90 *et seq.*) has emphasized the similarity between this family and the Parasemionotidae (some of the most advanced sub-Holostei, from the Eotrias), a similarity increased by the discovery of *Eoeugnathus*; it concerns particularly the cranial roof and does seem to constitute a link between the two orders. It is, however, the only one. There is no other sub-holostean (or palaeoniscoid) family known with corresponding resemblances to, say, the Pholidophoridae or Semionotidae, so that it remains doubtful whether the four other groups originated from an earlier pre-parasemionotid stock, or whether their unknown ancestors and the parasemionotids had a joint origin in the palaeoniscoids, or whether the Holostei are polyphyletic in a fairly sweeping sense and all the groups have arisen independently from a palaeoniscoid or very early sub-holostean stage. When their widely separate appearances in time are considered, the non-convergence of the earliest known forms, the fact that earlier offshoots of the palaeoniscoids (e.g. *Aeduella*, Westoll, 1937) have occasionally developed a free narrow operculum and vertical jaw suspension, and lastly the lack of any known ancestor sub-holostean ancestor for groups II–V, it may well be that the last suggestion of polyphylogeny will be preferred.

To be balanced against this is the impressive conservative homogeneous structure of the group—not so significant perhaps in the skull roof or mandible, for, the bones of the snout excepted, the former is not very different in all Actinopterygii, even the palaeoniscoids, and the latter is partially dependent upon mechanical necessities. But the neurocranial pattern is characteristically holostean and not general actinopterygian, and is of all structures the least subject to mechanical or adaptive influences; the bones of the postorbital section are very alike where they are known (those of the orbital surface are not shown in Fig. 12 but differ even less than the others). The pre-Jurassic fish faunas have yielded very few neurocrania, and nearly all are of one solid piece, except for a few rather aberrant derivatives of the palaeoniscoids. One may suppose therefore that the holostean ancestor or ancestors had a solid braincase, the embryonic ossifications fusing in the adult, but that in the holosteans themselves usually remaining separate, or only fused in some regions, such as the occipital. This is the breakdown in neurocranial structure discussed at some length by several authors, especially Stensiö (1932); the great resemblances in bone pattern resulting from it leads to the following conclusion: either one may consider the Holostei to be a self-contained natural order (though of largely unknown ancestry) the various groups of which show some divergence as a result of their subsequent evolution, or the resemblances are the result of convergence. Such a far-reaching convergence, without any evidence of adaptive or mechanical stimulus to favour it, seems to me unlikely, but it is fully admitted that the evidence is at best circumstantial.

The dividing line between the Holostei and Teleostei is clearly only arbitrary, the teleost line of evolution appearing with *Pholidophorus*. Smith Woodward (1895) emphasized this when he separated this line from the rest of the Jurassic holosteans as the Isospondyli, which also included the primitive Teleostei. If the higher bony fishes had only recently been investigated and classified, *Amia* and *Lepidosteus* should also form separate groups ranking on equal terms with the teleosts, and the present divisions would be indefensible. But if the object of a classification is to be useful, tradition has some claim in the use of well-known terms; all the divisions of the Actinopterygii are probably in part structural grades, so that, always provided that due definitions of the traditional terms are given, it seems well to let them stand.

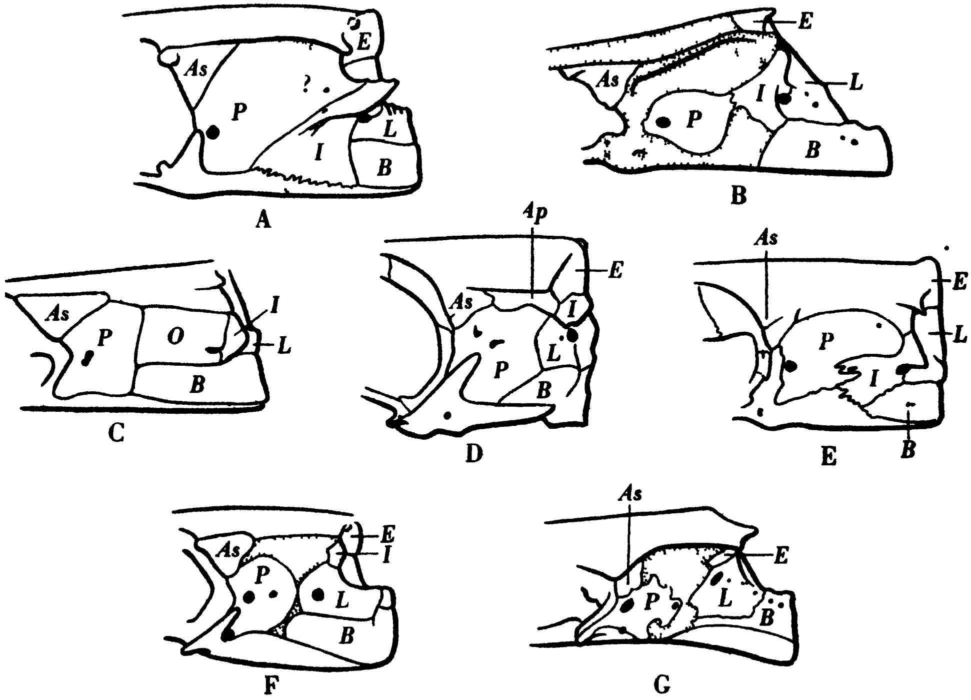

Fig 12 Holostean neurocrania in side view, slightly diagrammatic A, *Caturus*, B, *Amia*, C, *Hypsocormus*, D, *Leptolepis*, E, *Aspidorhynchus*, F, *Lepidotus*, G, *Lepidosteus* *Ap*, autopterotic, *As*, autosphenotic, *B*, basioccipital, *E*, epiotic, *I*, intercalar, *L*, lateral occipital, *O*, 'opisthotic', *P*, prootic, cartilage stippled

IV. SUMMARY

1. The Holostei are first found in the Permian; they are rare in most of the Trias but increase rapidly in the uppermost beds, becoming dominant in the Jurassic and Lower Cretaceous, after which they decline rapidly. The only living representatives are *Amia* and *Lepidosteus.*

2. As an order they are easily recognizable and have, with a very few exceptions, a number of 'stable' structural points in common, especially concerning skull roof, palate, mandible, fin position and structure. Inside the order five groups are distinguished, particularly on the maxilla, supramaxilla, cheek bones and neurocrania.

3. Groups I, II and V show some evolutional progression in the following series:

I: *Caturus, Megalurus, Sinamia, Amia.*
II: *Lepidotus, Lepidosteus.*
V: *Pholidophorus, Leptolepis,* primitive teleost.

4. Some adaptive radiation imposed on the inherited variations has produced the following types: fast predacious carnivores, slow bottom-living mollusc-eaters (including deepened fishes), small plankton-eaters, 'sword-fish' types.

5. Only the Eugnathidae can be traced back to probable sub-holostean ancestors, the Parasemionotidae. A monophyletic origin for the holostean groups is suggested by their homogeneous structure: a polyphyletic origin by their distribution in time and isolation from each other.

6. Brough's divisions of the Actinopterygii are upheld, while noting that they are probably in part structural grades; the division between the Holostei and Teleostei is arbitrary but convenient.

V. ACKNOWLEDGEMENTS

Some of the holosteans, especially the Macrosemiidae and Aspidorhynchidae, have been revised from material in the British Museum (Natural History); I wish to thank Mr W. N. Edwards and Dr E. I. White for access to their collections, and also for the loan of the original of Fig. 8 A. Figs. 7, 8 B and 9 are reproduced by the kind permission of the Trustees of the British Museum, and Figs. 4 B and 6 B by that of the Council of the Palaeontographical Society.

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THE EFFECTS OF HOSTS UPON THEIR INSECT PARASITES

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(*Received* 30 *December* 1940)

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I. INTRODUCTION

It would require a considerable book to review adequately the various effects of parasites on their hosts. The subject is one of practical importance and must have attracted attention, at any rate with regard to the parasites of man, from very ancient times. Indeed, some account of their effect upon their hosts is essential for any consideration of the economy of parasites, and the subject is therefore implicit in the writings both of the early naturalists and of medical and veterinary parasitologists. A more specifically zoological interest in it was aroused by Giard in 1886 when he first described, under the title of "castration parasitaire", the effects of Rhizocephala on their crustacean hosts. Since then, many investigators have concerned themselves directly with the problem. One has only to mention the work of Giard, Geoffrey Smith, Caullery, and Tucker on the influence of cirripede, bopyrid, and epicarid parasites on decapod Crustacea; of Wheeler and Vandel on the intercastes produced by nematodes in ants; of Pérez, Wheeler, and myself on the effects of stylopization on bees and wasps; and of Pantel, Kornhauser, Silvestri, Thompson, and Codreanu, among many others, on the effects of insect parasites on a variety of hosts, to indicate the wide and lasting interest that the subject has provoked.

How different is the state of the converse problem—that of the effects of hosts upon their parasites! It is not even obvious that there is such a subject, for the host is usually looked upon as the passive victim, influenced by, not itself influencing, the parasite. Nor has the problem when formulated any self-evident importance. For these reasons, perhaps, it has been almost completely neglected. I have not been able to find any major work on the subject so far as the parasitic worms, Crustacea and Mollusca are concerned. As for the parasitic insects, although there are several notes and a few papers dealing with particular manifestations of the phenomenon, the problem as a whole has never been subjected to any comprehensive treatment.

It is my purpose in the present review to provide a general account of the effects of hosts upon their parasites with reference to the parasitoid Insecta.

One preliminary explanation must be made. A parasite sometimes finds itself on a host that is totally unsuitable for it. Unless it is then able to change its host (and the majority of insect parasites cannot do so), it eventually dies. One might claim that the effect of such unsuitable hosts is to kill their parasites; and that this constitutes the most drastic of the effects I have chosen to discuss. On the other hand, one might hold that a completely unsuitable host is not really a host at all; and that the parasite dies, not as the effect of a host, but for want of one. This is a quibble that may for the present be left unresolved. In this article I shall be concerned with the morphological, physiological and behaviouristic characters that are impressed by the host upon the living parasite.

Table I. *Average size in mm. of three species of hosts and of individuals of* Telenomus ashmeadi *reared from them* (*after Morrill*, 1907)

Host species	*Pentatoma ligata*	*Euschistus servus*	*Thyanta custator*
Average diameter of host egg	1·01	0·88	0·75
Average width of head of parasite	0·60	0·53	0·45

II. EFFECT ON SIZE

The effect of hosts on the size of their parasites is the most obvious influence they exert and has naturally been the one most frequently commented upon. As early as 1844, Ratzeburg pointed out that *Pimpla examinator* varies in length from 1⅔ to 6 lines, and remarked that this depends on the size of the host on which the parasite developed. Similar observations and remarks are common in the entomological literature of the last forty years (e.g. Toyama, 1906, in *Tachina*; Shevirëv, 1913, in *Pimpla*; Altson, 1920, in *Alysia*; Willard, 1927, in *Microbracon*).

Rather more precise data were provided by Morrill (1907), who studied the proctotrypid *Telenomus ashmeadi*. He reared this egg-parasite on three species of pentatomid bugs of different sizes, and measured five male emergents from each host. His measurements, reproduced in Table I, show that the size of the emergent parasites, as represented by the width of their heads, was correlated with the size of the hosts in which they had developed.

In 1924 Mickel published an account of the mutillid wasp, *Dasymutilla bioculata*, which varies in length from 6 to 15·5 mm. He plotted the lengths of a large number of individuals and showed that they form a bimodal curve (Fig. 1). He then found that this species parasitizes two different genera of bembecid wasps, *Microbembex monodonta*, which ranges in length from 8 to 14 mm., and *Bembex pruinosa* which varies from 16 to 19 mm. By rearing the mutillid from each of these hosts, he was able to show that the smaller individuals develop at the expense of the smaller host while the larger ones develop on the *Bembex*.

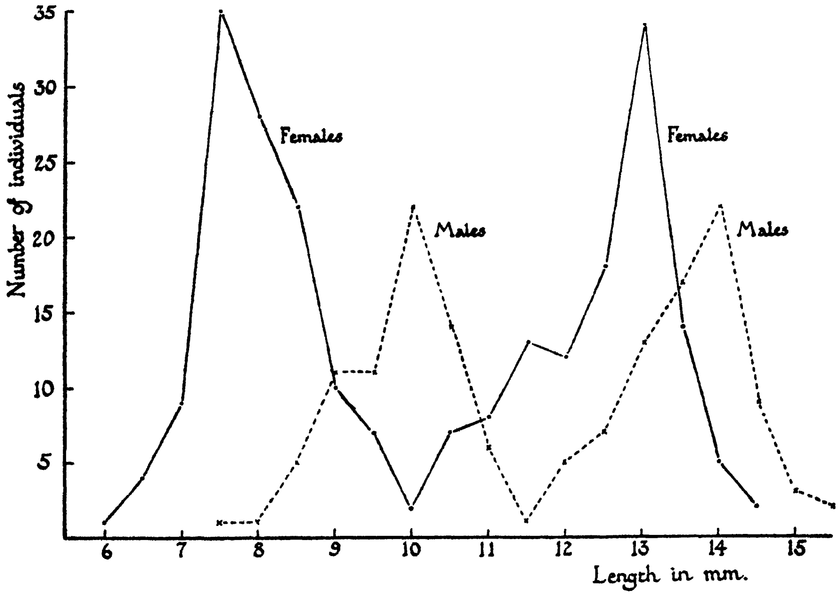

Fig. 1. The size groups of 150 males and 232 females of *Dasymutilla bioculata* (after Mickel, 1924).

A similar state of affairs exists in the braconid parasite *Opius melleus* which attacks the apple maggot, *Rhagoletis pomonella*, as well as the much smaller blueberry maggot, *R. mendax*. Lathrop & Newton (1933) have pointed out that the parasite is tending to develop a large and a small strain, and have published an excellent figure showing the great difference in size of parasites reared from the two hosts.

That the difference in size in such cases is a direct effect of the host, and is not due to the selection of large and small hosts by pre-existing strains of large and small parasites, can only be demonstrated by rearing the progeny of an individual parasite. For this reason, the data of Jackson (1937) on *Pimpla examinator* are especially interesting. A female of this ichneumonid, reared from *Abraxas grossulariata*, measured 10·7 mm. in length. Its largest female offspring, developed on

Pieris brassicae, was 14 mm. long; its smallest female progeny, reared on *Ephestia kuehniella*, was 7·1 mm. long, and its smallest male descendant, also from *Ephestia*, measured only 5·6 mm.

In the egg-parasite *Trichogramma evanescens*, also, the progeny of an individual parasite have been shown to vary in size according to the species of host on which they are reared (Salt, 1940). A female was allowed to oviposit in two or three eggs of each of three different species, and its progeny were reared and measured. Fifteen females were used, with the average result shown in Table II. In a particular case, illustrated by Fig. 2, the parent measured 0·40 mm. in length. Its smallest female offspring, reared from an egg of *Sitotroga cerealella*, was 0·34 mm. long; each of its three female progeny from eggs of *Ephestia kuehniella* measured 0·46 mm.; and its largest offspring, a solitary female from an egg of *Agrotis c-nigrum*, attained a length of 0·57 mm. The correspondence between the size of these parasites and that of their hosts is indicated by the figure.

Table II. *Average size in mm./10 of individuals of* Trichogramma evanescens *reared from three different species of hosts (after Salt,* 1940)

	No. measured	Size of parasite				Size of host	
		Length	Width of			Length	Diam.
			Head	Thorax	Abdomen		
Parents	15	4·1	1·8	1·4	1·5	5·5	2·4
Progeny from:							
Sitotroga	38	3·9	1·7	1·4	1·4	4·4	2·3
Ephestia	40	4·7	2·1	1·7	1·7	5·7	3·6
Agrotis	49	5·1	2·4	1·9	2·0	5·6	6·5
Agrotis (solitary)	4	5·8	2·8	2·2	2·5	5·6	6·5

These data all show that the size of a parasite is influenced by different species of hosts. It remains to discover whether the size of the parasite can be influenced by different individuals of one species of host. There are many statements in the literature that this is so, but apparently the only actual measurements are those relating to *Trichogramma evanescens* (Salt, 1940). A female of this egg-parasite was allowed to lay in eggs of *Sitotroga cerealella* that had been selected for their smallness, and then in others that were chosen for their large size. The hosts, the parent, and the female progeny were measured, and the experiment was repeated several times. The measurements, which are published in full in the paper cited, and the averages, which are set out in Table III, show that the size of the parasite is influenced by the varied size of individual hosts within one species.

This result suggests that in general the effect of the host on the size of its parasite is not due to differences in the kind of food provided by various hosts but simply, as one would expect, to differences in its amount. The suggestion is supported by a consideration of gregarious parasitoids. In species that have this habit, a number of individual parasites share a single host. It has often been observed that the more of them there are together, the smaller they will be (e.g. Faure, 1923; Jacobi, 1939);

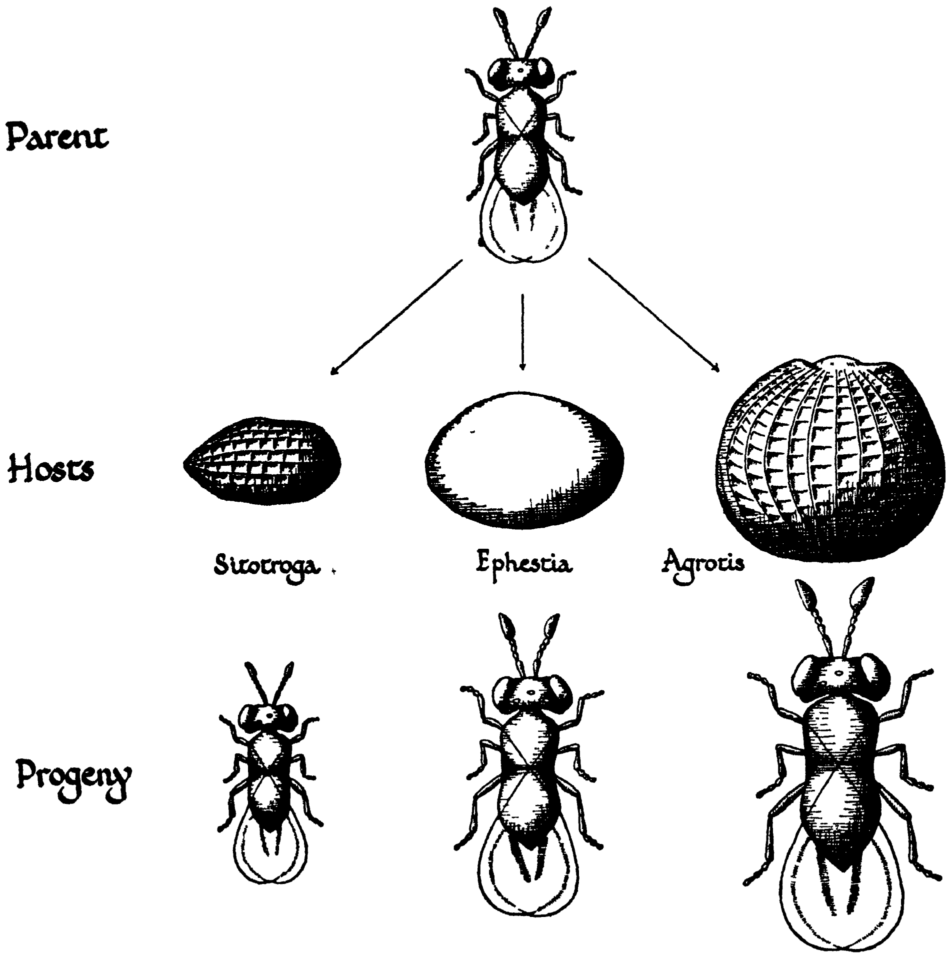

Fig. 2. Relative sizes of a female *Trichogramma evanescens* and its progeny reared from three different genera of hosts.

Table III. *Average size in mm./10 of eleven large and eleven small eggs of* Sitotroga cerealella *and of the individuals of* Trichogramma evanescens *reared from them* (*after Salt*, 1940)

	Hosts		Parasites			
				Width of		
	Length	Diam.	Length	Head	Thorax	Abdomen
Small hosts	4·8	2·2	4·0	1·7	1·3	1·4
Large hosts	5·9	2·4	4·8	1·9	1·5	1·6

and they are known to range very greatly in size. Since this variety is due less to the size of the host than to the number of parasites sharing it, the gregarious parasitoids need not be considered in detail in this review. They do, however, provide additional evidence that in the experiments described above the parasites varied in size as a result of the amount, rather than the kind, of food provided by the different hosts. In short, the effect of the host on the size of its parasite is merely a special case of the general problem of feeding and growth.

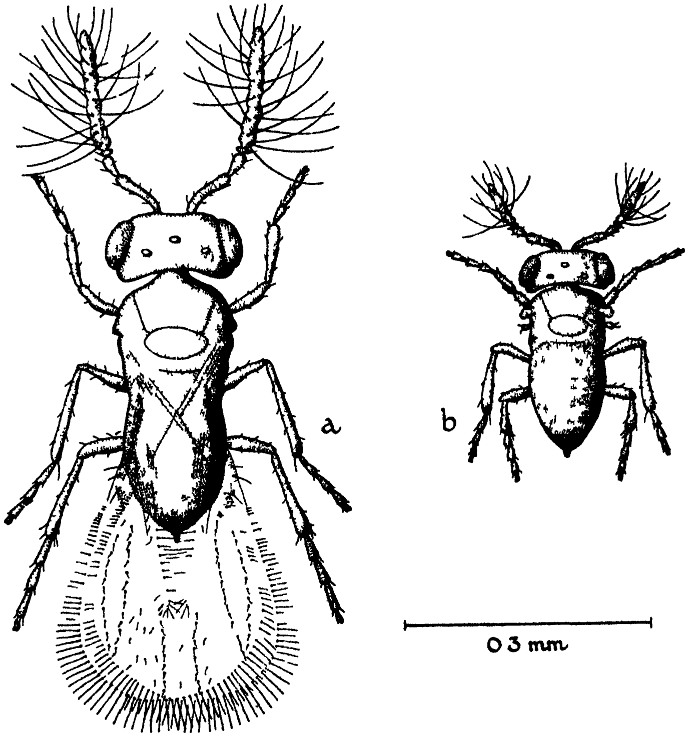

Fig. 3. *Trichogramma evanescens*. *a*, normal male; *b*, runt produced as a result of undernourishment.

When the amount of food provided by the host is sufficient but scanty, the parasite emerges, as we have seen, as a normal but tiny individual. When the food is quite inadequate, the parasite is unable to develop and, of course, dies. On the boundary between these two conditions, the amount of food may be just enough to allow the parasite to survive, but insufficient to allow its perfect development. In this case, abnormal individuals known as runts are sometimes produced (Fig. 3). The abnormality takes the form of a stunted development of various parts of the body accompanied by a failure to develop or expand the wings. Runts have not often been described among parasitic insects because they are not active enough and do not survive long enough to be found in nature; but they occur among the

Diptera (*Thrixion*, Pantel, 1910) and are well known in the Hymenoptera (*Trichogramma evanescens*, Zulueta, 1928; Salt, 1940; and *T. semblidis*, Salt, 1939). The formation of these runts is the final stage of the reduction in size of a parasite under the influence of a small host.

III. EFFECTS ASSOCIATED WITH THE EFFECT ON SIZE

(1) MORPHOLOGICAL

(*a*) *Size and proportion of parts*

The size of the several parts and appendages of a parasite varies with that of the complete animal and will therefore be influenced by the host just as the whole animal is influenced. For example, Oldroyd & Ribbands (1936) have shown that the length of the wings in *Trichogramma evanescens* and the number of macrotrichia forming the rows on the wings (cf. Fig. 4) are closely correlated with the size of the whole parasite; and I have observed (Salt, 1940) that the number of long hairs on the male antennae also varies with the size of the individual. No doubt other similar examples could easily be found. Cases in which a particular structure exhibits heterogonic growth would be particularly interesting but have apparently not yet been described.

Such effects as these are of special interest to the systematist. Pantel (1910, p. 176) has already pointed out that the variety *barretii* of *Thrixion halidayanum* was established by Tavares for specimens differing from the typical form only in that they had been stunted by development in too small a host. *Meigenia floralis* variety *major* is an example of the converse effect. Oldroyd & Ribbands (1936) have shown that a character used by Ellinger and Sachtleben to distinguish species of *Trichogramma* varies with the size of the host and is therefore invalid for that purpose. The notorious difficulty attending the classification of parasitoid insects is partly due to wide variability, which may often be attributable to the influence of different host species.

(*b*) *Presence and absence of wings*

A number of parasitic insects, principally among the parasitoid Hymenoptera, occur in two forms, one winged, the other apterous or brachypterous.[1] Sometimes only one sex is dimorphic; sometimes both. A genetic explanation has been suggested in one case (*Cephalonomia*, van Emden, 1931), but in the great majority of instances the factors controlling the dimorphism are quite unknown. In a few cases there is some evidence that the host, through its effect on the size of the parasite, may be responsible.

The male of *Pezomachus flavocinctus*, for example, occurs in winged and wingless phases. According to Strickland (1912, p. 131) the apterous males "are much smaller than the winged forms which would suggest that their apterous condition is in some way connected with an insufficiency of food". Both sexes of the braconid *Sycosoter lavagnei* are dimorphic in respect of the wings. Of this species, Lichtenstein & Picard (1917, p. 457) write: "les ailés sont d'une taille supérieure à celle des aptères. On pourrait donc admettre qu'une nutrition insuffisante en réduisant la taille de l'adulte, agit aussi en entravant le développement des disques alaires."

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[1] There are apparently no established cases among the parasitoid Diptera. The apterous specimens of *Thrixion halidayanum* variety *barretii* reared by Tavares (1906, p. 90) and mentioned by Pantel (1910, p. 177) are certainly to be classed as runts—individuals so small and weak that they were unable to expand their wings.

The above examples provide evidence, but not proof, that the size of the host, acting through its influence on the size of the parasite, sometimes controls the development of the wings in dimorphic forms. A step towards the required demonstration was taken by Thompson (1923) who reared two male progeny from one female of *Pezomachus sericeus*, one on a large host, a cocoon of *Camponotus*, and the other on a small host, a cocoon of *Apanteles*. The male that emerged from the former was large and fully winged; that developed on the latter was small and completely apterous. An accident to the parent female unfortunately prevented the continuance of this interesting experiment, and the result is susceptible of more than one interpretation. Nevertheless, as it stands, it clearly indicates the line that the required demonstration might take.

(2) PHYSIOLOGICAL

It is well known that certain physiological characteristics, such as fecundity, longevity and vigour, are correlated with larval feeding and adult size in many insects (Glaser, 1923; Weidling, 1928). It may be expected, therefore, that the host, through its effect on size, may also influence its parasite in these correlated physiological characteristics. Unfortunately, although the assumption is plausible, or perhaps because it is so plausible, the actual data to demonstrate it in parasitic insects are few and unsatisfactory. Such as they are, they can be arranged as follows.

(*a*) *Developmental period*

Certain variations in the developmental period of *Trichogramma evanescens* are perhaps to be attributed to the different sizes of the hosts from which the parasite was reared. It has been observed that individuals developing in eggs of *Ephestia* emerge sooner than individuals reared under identical conditions in eggs of *Agrotis* and *Sitotroga* (Salt, 1940). It has also been noticed that when two or three parasites share one egg of *Agrotis*, they emerge much earlier than solitary parasites from *Agrotis* and at nearly the same time as solitary parasites from *Ephestia*. Since other factors, such as the hardness of the shell through which the emerging parasites have to bite their way, cannot be entirely ruled out, the observations do not permit a definite conclusion, but it seems likely that there is an optimum amount of food for the rapid development of *Trichogramma*, and that it is near the amount provided by one egg of *Ephestia* or by one-third of an egg of *Agrotis*. If this conclusion is correct, the size of the host can be said to influence the developmental period of the parasite.

(*b*) *Mating*

According to Hase (1925, p. 198) the range of size in *Trichogramma evanescens* is so great that normal copulation cannot take place between the largest and the smallest individuals.

(*c*) *Fecundity*

There appears to be no formal demonstration of the effect of the host on the fecundity of its parasite. Roubaud (1924, p. 236) says that the chalcid *Pachycrepideus* has 2, 7 or 8, or 20 ovarioles, according to its size, which is according to its nourishment as a larva. In another chalcid, *Eupteromalus nidulans*, Proper (1931, p. 44) observed the fecundity of sixteen females and says that "in general, the smallest individuals laid the fewest eggs, and the largest laid the most". Taylor (1937, p. 194) finds that females of *Pleurotropis parvulus* normally have six ovarioles, but small females may have only three, two in one ovary and one in the other. Large females lay 60–85 eggs; medium-sized females 35–67 eggs; and small females only 18–32 eggs. He gives similar data (p. 156) for *Dimmockia javanica*. Among the parasitic flies, *Zenillia libatrix* shows great variation in the number of ovarioles and eggs, according to Dowden (1934, p. 108). Large individuals have about a hundred ovarioles in each ovary and produce over 2400 eggs; while small females may have as few as sixty-one ovarioles on each side and produce only 820 eggs. All these species, however, are gregarious parasitoids in which size is only to a slight extent a function of the size of the host. They cannot, therefore, provide a clear demonstration of the effect of a host on the fecundity of its parasite.

Flanders (1935, p. 177) says that large individuals of *Trichogramma* may be over five times as prolific as small ones, but it is not clear whether he is referring to solitary parasites or to specimens reared gregariously. From a table I have published (1935, p. 417) it can be taken that solitary individuals of *Trichogramma evanescens* reared from eggs of *Ephestia* laid an average of 23·1 eggs each, while the smaller females from eggs of *Sitotroga* laid only 19·8 eggs each; but here again the data are unsatisfactory for our present purpose because they refer only to an 8 hr. oviposition period.

(*d*) *Vigour*

It is very likely that in many parasitic insects, large individuals are able to fly farther, move faster, find more hosts, and so on, than small individuals of the same species; but there appear to be very few published facts. One example is provided by Flanders (1935, p. 177) who says that "Large specimens of *Trichogramma* are able to oviposit in larger hosts than are small specimens", and mentions the inability of small individuals to oviposit in eggs of *Pachysphinx modesta*, which are readily parasitized by large ones.

If the time taken by a parasite to pierce the hard shell of its host be taken as a measure of its strength, large females of *Trichogramma evanescens* are certainly stronger than small ones. Large, medium-sized, and small females were presented successively with eggs of four different species of moths and were allowed to parasitize two of each of them (Salt, 1940). The results of five such experiments, summarized in Table IV, show that large parasites emerged from *Barathra* penetrated the hosts more quickly than medium-sized parasites from *Ephestia*, and much more quickly than small ones reared from *Sitotroga*.

If, further, the time required for a parasite to perform a succession of such acts of strength may be taken as an indication of its vigour, large parasites are more vigorous than small. For females of *Trichogramma* to find, examine, penetrate, and parasitize ten hosts required, on an average, 66 min. when the parasite was a small individual reared from *Sitotroga*, but only 37 min. when it was a large female emerged from *Agrotis* (Salt, 1940).

Table IV. *The average time required by females of* Trichogramma evanescens *from three different hosts to drill through the chorion of four species of host eggs* (*from Salt*, 1940)

Parasite from	*Sitotroga*	*Ephestia*	*Barathra*
Average length in mm./10 ...	4·2	4·7	5·2
Time required to drill through:			
Sitotroga	1 min. 14 sec.	1 min. 1 sec.	50 sec.
Ephestia	44 sec.	36 sec.	32 sec.
Agrotis	1 min. 34 sec.	1 min. 31 sec.	1 min. 2 sec.
Hydriomena	13 min. 52 sec.	10 min. 30 sec.	7 min. 14 sec.

(3) BEHAVIOURISTIC

In one case, at least, the behaviour of a parasite is known to be affected by its size, and therefore by the size of its host. That is in the selection by females of *Trichogramma evanescens* of hosts in which to deposit their own progeny. Flanders (1935, p. 177) briefly mentioned this fact in the following words, "Large specimens [of *Trichogramma*] tend to ignore hosts of less volume than their own bodies. Solitary specimens from *Estigmene* eggs confined with *Sitotroga* eggs did not oviposit although their parents were reared from *Sitotroga* eggs."

Experiments that I have recently published (Salt, 1940) demonstrate the point in greater detail. When large females of *Trichogramma evanescens* reared from eggs of *Agrotis* were placed with small eggs of *Sitotroga* and kept under observation, they passed from egg to egg, clearly seeking hosts but completely rejecting the small eggs available to them. Moreover, when large and small females were put in separate dishes for several hours with small eggs of *Sitotroga*, only one of the large females parasitized any of the hosts, but all of the small females accepted and parasitized them. Still further, when large females were kept with small hosts throughout their adult life, one parasitized four of the small hosts (less than one-tenth of the number it could have parasitized), the others would not attack any and died without leaving any offspring. In these experiments, the large hosts must be considered to have influenced the behaviour of the large parasites that emerged from them.

IV. APPARENT EFFECT ON SEX

Under this heading we must briefly consider a number of observations which appear to fall within the scope of this review although, as will be shown, they have no real place in it. Several authors have observed, namely, that large hosts tend to produce an unduly high proportion of female parasites and small hosts an excess of male parasites. The phenomenon has been observed in the Ichneumonidae (Shevirëv, 1913), the Braconidae (Holdaway & Smith, 1932), the Chalcididae (Taylor, 1937), and the Scoliidae (Brunson, 1937). All the known cases have been usefully brought together and discussed by Clausen (1939) and need not be reviewed here.

Of the fact—that in several species of parasitic Hymenoptera mostly female parasites emerge from large host individuals and mostly males from small hosts—there can be no doubt. But the causes of the phenomenon are less certain. Several explanations have been suggested, of which the most obvious and apparently straightforward is that the size of the host influences the sex of the parasite emerging from it.

If this were so, the matter would be of particular interest in the present review. But such a hypothesis is so completely at variance with all that is known of sex determination among insects that very strong evidence indeed would be required to support it. Instead, there is no evidence for it whatsoever; and all of the known cases can be reasonably explained on other grounds. We can therefore conclude that no real effect of the host on the sex of its insect parasite has yet been established; and the apparent relevance of the observations to the subject under review simply disappears.

V. EFFECT ON MORPHOLOGY

The morphological characters of an animal species are usually considered to be rather rigidly fixed, or at most to vary within somewhat narrow limits. Nevertheless, structural differences so profound that they would be ample for the separation not only of species but even of genera have recently been found to be due to the influence of a particular host on the males of *Trichogramma semblidis*.

This parasite has been known, ever since it was originally described by Aurivillius in 1898, to have a dimorphic male. One of the forms is winged, the other apterous (Fig. 4); but this, although it is the most noticeable, is by no means the only difference between them. The two forms also differ very markedly in the segmentation of the antennae; in the occurrence of long setae on the flagellum of winged males; in the shape of the various segments of the legs (Fig. 5); and, internally, in the presence in winged males of an enormously developed post-phragma which is lacking in the apterous form.

It is important to observe at the outset that the apterous males of *T. semblidis* are large and perfectly formed individuals which must not be confused, as so many authors have confused them, with the runts produced in *T. evanescens* by undernourishment (compare Figs. 3 *b* and 4 *b*). Moreover, the two forms are entirely discrete; there are no intermediates between them (Salt, 1939). In short, in this species there is a true and fundamental dimorphism of the male sex.

Marchal (1936, pp. 501–14) was the first to suggest that the dimorphism of *T. semblidis* is controlled by its host. Unfortunately, owing to his confusion of two distinct species of parasites, *evanescens* and *semblidis*, and his consequent failure to distinguish clearly between the normal apterous males of *T. semblidis* and the abnormal, nearly apterous runts of *T. evanescens*, he was unable to carry his suggestion to the stage of proof. That was done by quite independent and almost contemporary work of my own (1937).

The fact is that whether the winged or the apterous form of *T. semblidis* shall appear is almost completely determined by the species of host in which the parasite

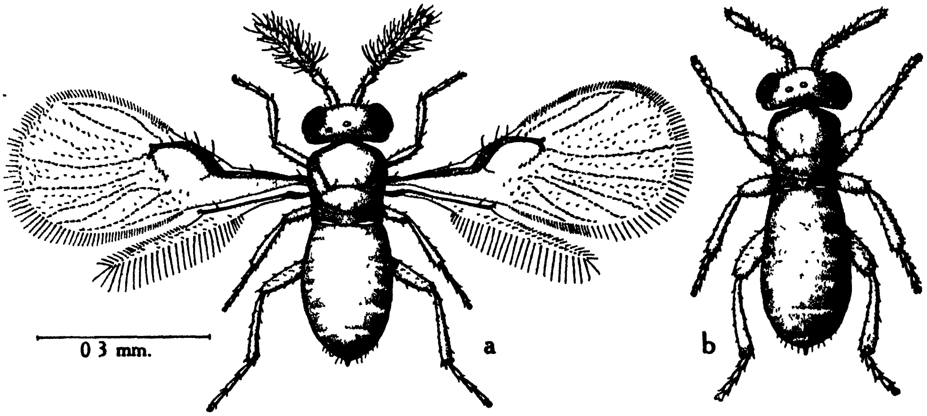

Fig. 4. *Trichogramma semblidis*, male. *a*, winged form reared from an egg of a moth; *b*, apterous form reared from an egg of *Sialis lutaria*.

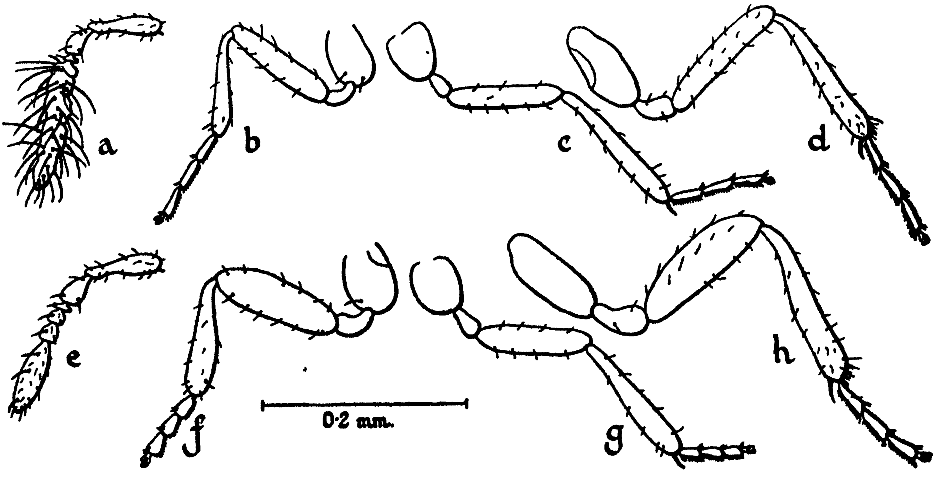

Fig. 5. *Trichogramma semblidis*, male. *a*, *b*, *c*, *d*, antenna, fore-, middle-, and hind-leg of the winged form; *e*, *f*, *g*, *h*, same of the apterous form.

develops—winged males are produced when the parasite is reared on eggs of Lepidoptera, apterous males when it develops in eggs of the neuropteron, *Sialis lutaria*. The evidence for this statement is now overwhelming. Out of many thousands of individuals reared in the course of three years' work, 3617 normal males have been carefully examined. Of these, thirty, all of the winged form, were reared from two species of *Chrysopa*. The remaining 3587 were all reared either on

Sialis lutaria or on one of the three species of moths, *Sitotroga cerealella, Ephestia kuehniella*, and *Barathra brassicae*. The distribution of apterous and winged forms from these four hosts is shown in Table V, where it will be seen that among the 3587 individuals there are only nineteen exceptions to the rule that the apterous form comes from eggs of *Sialis* and the winged form from the eggs of moths.

These gross figures are further supported by breeding experiments extending through sixty-five generations of the parasite. Small-scale experiments using the progeny of isolated females fertilized by their brothers and carried back into *Sialis* eggs after one or two generations in *Ephestia* (Salt, 1937, p. 547) show conclusively that no genetic explanation will fit the case. Large-scale experiments in which members of a pure strain of the parasite were reared on *Sialis* after thirty-two and sixty-four continuous generations on *Sitotroga* (Salt, 1939, p. 518) show that the dimorphism remains latent through as many as sixty-four generations and yet is immediately expressed when the appropriate host again exerts its influence.

Table V. *The emergence of* 3587 *males of* Trichogramma semblidis *from eggs of three species of moths and from eggs of the alder-fly,* Sialis lutaria (*from Salt,* 1939)

Host	Apterous males	Winged males
Sitotroga cerealella	2	803
Ephestia kuehniella	11	986
Barathra brassicae	0	45
All three moths	13	1834
Sialis lutaria	1734	6

How the host is able to determine the form of the emergent parasite is a problem that has not yet been attacked experimentally, but it seems clear from a variety of facts which I have reviewed elsewhere (1937) that it is the different nourishment of the parasite in the one host and the other that controls the appearance of the different forms. Why there should be exceptions to the general rule (nineteen out of the 3587 cases in Table V) is also a question that has yet to be answered, although the divergence is so small (0·53 %) that it cannot affect the main conclusion.

The figures in the two papers cited are so large, and the rearing experiments so extensive, that they allow no doubt that in the case of the dimorphic males of *Trichogramma semblidis* which form of the parasite shall emerge is almost completely determined by the host. To the present, this striking effect of the host on the morphology of its parasite remains unique.

This result, like that on p. 245, carries a warning for systematists. If both sexes of *T. semblidis* had been dimorphic, instead of only the males, the two forms would no doubt have been put into different genera. There, like the alternate generations of Cynipidae, they might well have remained for many years until a lucky rearing in the correct host revealed their true relation. There can be no certainty that our present classification does not contain such forms, dimorphic in both sexes and with the phenotypes determined by the host, masquerading as perfectly distinct and widely separated species.

VI. EFFECTS ON PHYSIOLOGY

(1) RATE OF DEVELOPMENT

The life history of an insect parasitoid is often closely synchronized with that of its host. In many cases this is due to physical factors, especially temperature, acting more or less equally on the two animals. In some cases it is known to be due to an effect of the parasite stimulating the host to premature or to delayed development (cf. Varley & Butler, 1933). But there remain instances where it appears certain that the conformity is brought about by the influence of the host on the development of its parasite.

That this might be so seems to have been first suggested by Pantel (1910, p. 173). In his account of the larvae of parasitoid Diptera, he includes the host among the external conditions that influence the duration of the larval stage; and instances the two tachinid flies, *Compsilura concinnata* and *Tricholyga major*. The former is said to mould its development on that of its host, growing rapidly in *Acronycta* and *Vanessa*, where it is full grown in some weeks; and slowly in *Pieris*, where its development may occupy several months. In the case of *Tricholyga*, the parasite also develops rapidly on *Vanessa* but slowly on *Macrothylacia rubi*.

Only two years later, a similar effect was described in the parasitic Hymenoptera when Smith (1912, p. 44) showed that the life history of the chalcid *Perilampus hyalinus* is modified by the host it inhabits. The larva must emerge from its host at the time of pupation. If it attacks *Limnerium validum*, which does not pupate until the spring, the planidium remains endoparasitic and undeveloped during the winter; if it attacks *Varichaeta aldrichi*, which pupates in the autumn but does not emerge until the spring, the parasite becomes ectoparasitic in the autumn but develops no further until the next season; but if the parasite finds itself in a host, such as two other species of *Limnerium* and two braconids, which not only pupate in the autumn but also proceed to immediate emergence in the same season, the parasite likewise develops at once and hibernates as a full-grown adult. The life history of *Perilampus cuprinus* (Smith, 1912, p. 66) is similarly affected by its various hosts.

Another case in the Hymenoptera is that described by Faure (1926, p. 48) in the braconid *Apanteles glomeratus*, a common parasite of the cabbage caterpillar, *Pieris brassicae*. This host usually pupates in the autumn, and the larvae of *Apanteles* then become full grown and leave it just as it prepares to pupate, spinning about its remains the familiar cluster of cocoons in which they hibernate as prepupae. If, however, the host is not ready to pupate in the autumn and hibernates as a larva, as *Pieris brassicae* does occasionally and as another host, *Aporia crataegi*, does commonly, the *Apanteles* then hibernate as young larvae inside the host caterpillar.

In some cases this influence of the host acts in such a manner as to determine whether the parasite shall have one or more than one generation a year. According to Faure (1924, p. 72), *Bracon glaphyrus* normally attacks larvae of the weevil *Baris laticollis*, which is univoltine; and in that host the parasite has one generation a year. If it accidentally lays in *B. chlorizans*, which is partially bivoltine, it has two generations in a year. Commenting on this, Faure (1926, p. 196) writes: "Il apparait que, avant tout, la biologie des Hyménoptères parasites est dominée par celle de leur hôte." Dowden (1935, p. 517) has made a similar observation on *Brachymeria compsilurae*. This chalcid attacks both *Sturmia scutellata*, which has one generation a year, and *Compsilura concinnata*, which may have two or three generations. When it parasitizes the former host, *Brachymeria* remains in the larval stage during the winter and has only one generation in a year; but when it attacks *Compsilura*, the parasite may itself have two or three generations a year. ✓

According to Thompson & Parker (1928, p. 28) the ichneumonid *Dioctes punctoria* is similarly influenced by univoltine or bivoltine strains of its host, *Pyrausta nubilalis*, and has one generation a year in some districts, two in others. In transitional areas where a few corn-borers pupate very late in the season, thus giving rise to a partial second generation, some *Dioctes* also mature and emerge in the autumn. The authors write: "It thus seems probable that the condition of the host has a direct influence upon the development of the parasite...."

These and other similar instances to be found in the literature are observations made in the course of more general studies. In no case was their author concerned to study the phenomenon they exhibit specifically and in detail. It remained for Marchal (1927, 1936) to deal with the problem deliberately and to demonstrate clearly the effect of a diapause of the host on the development of its parasite.

Marchal studied an egg-parasite, *Trichogramma cacaeciae*, which in France is normally found attacking eggs of *Cacoecia rosana*, a tortricid moth; but which in the laboratory will attack other hosts, including eggs of the cabbage moth, *Mamestra brassicae*. The life cycle of the normal host, *Cacoecia*, includes only one annual generation. The moths emerge in June and early July, and lay their eggs in the course of the latter month. These eggs almost immediately enter an embryonic diapause which does not come to an end until the following March. Then the embryo develops rapidly. The eggs hatch in April and the larvae feed until June. The pupae are formed early in June and develop into the next generation of moths at the end of that month and during the first part of July.

Trichogramma cacaeciae has two generations to each one of *Cacoecia*. Eggs are laid in the fresh eggs of the moth in July. Within a few days the parasite larva devours the contents of the host egg, and then itself enters a larval diapause from which it is not released until early spring. In March it pupates and later in that month or early in April it emerges as an adult. The females of this generation immediately attack the still unhatched hosts laid the previous year; and their progeny, developing in six or seven weeks, produce the second generation of parasites late in June or early in July.

The life cycle of the *Trichogramma*, then, is very nicely fitted to that of the *Cacoecia* (Fig. 6). One of its generations is passed in the rapid development of which all the species of *Trichogramma* seem capable. The other generation extends over a period of nine months, during seven of which the larval parasite is in a state of diapause.

But this life cycle is not essential to the parasite. If the adults emerging in June are allowed to attack eggs of *Mamestra brassicae*, which have no embryonic diapause, their progeny develop immediately and produce a new generation of parasites within a month. This extra generation can be supplied with fresh eggs of either *Cacoecia* or *Mamestra*. If it is put with the former, the progeny enter the usual diapause and do not emerge until the following spring. In effect, therefore, an extra generation on *Mamestra* has been interpolated into the usual life cycle. But if the extra generation of parasites is put with eggs of *Mamestra*, another rapid generation is passed, for in this host the development of the parasite is never delayed and five or six successive generations can be reared from it between July and the onset of winter.

Marchal's series of rearings permit no doubt that it is the diapause of the host that retards the development of the parasite. As we have seen, if the host is in active development, as are the eggs of *Cacoecia* in April or the eggs of *Mamestra* at any time, the parasite develops without delay. But the active development of the host is unnecessary. Eggs in course of development that are killed by heating and then parasitized also allow an immediate development of a parasite. If, however, the host is in a diapause, that diapause is communicated to the parasite and its development is inhibited.

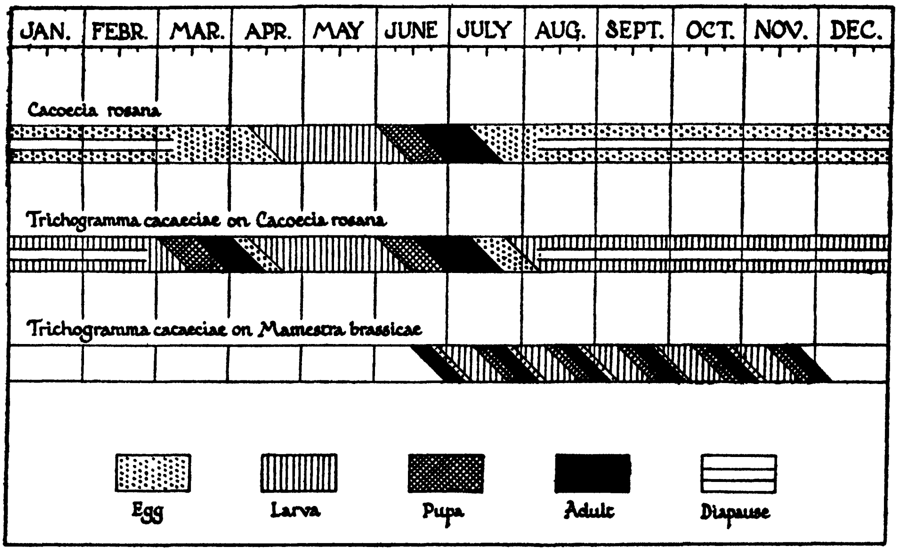

Fig. 6. The effect of the diapause of *Cacoecia rosana* on the life cycle of its parasite *Trichogramma cacaeciae*.

The results obtained by Marchal can hardly be explained other than by assuming that some inhibitive substance in the diapause eggs of *Cacoecia* is ingested by the *Trichogramma* and acts upon the parasite as it would upon the host. Such a view recalls the suggestion of Roubaud (1924, p. 239) who tried to explain the synchrony of the parasitic fly *Anacamptomyia* with its host, *Belonogaster*, in a similar way. He writes: "Il convient, en effet, de considérer les jeunes larves de Tachinaires

pendant toute la période où l'organisme de l'hôte a conservé une croissance active, comme dominées par les sécrétions internes de cet organisme-hôte...elle-même."

Marchal's work throws light upon numerous other cases in the literature which, for want of a better explanation, have hitherto been attributed to the perversity of biological material. Pantel (1910, p. 174) found that from a lot of chrysalids of *Pieris* collected in autumn, some butterflies emerged that year, others the following spring. A proportion of the chrysalids were parasitized by *Compsilura concinnata* and the parasites emerged, like their hosts, some in the autumn and others in the spring. Thompson (1910, p. 292) had a similar experience with the same tachinid fly reared on *Hyphantria textor*. More recently, Webber (1937, p. 148) has published data (Table VI) showing that when *Compsilura* attacked hosts late in the season all the progeny hibernated as larvae, whereas from attacks made on the same host species only a fortnight earlier a high proportion of the progeny developed immediately. These observations are perfectly understandable on the basis of Marchal's work, if the hosts that were still in active development at the earlier date had entered their diapause by the time of the later attacks.

Table VI. *Development of* Compsilura concinnata *after autumn parasitization (from Webber,* 1937)

Date of parasitization	No. of parasites that developed immediately	No. of parasites that hibernated as larvae	Parasites that went into hibernation %
Of *Hyphantria cunea*:			
30–31 Aug.	25	6	19
13 Sept.	0	10	100
Of *Euchaetias egle*:			
6 Sept.	79	0	0
1–8 ,,	6	1	14
15 ,,	0	10	100
18 ,,	0	9	100
Of *Symmerista albifrons*:			
14–21 Sept.	1	10	90
19–28 ,,	1	13	92
9–11 October	0	1	100

It may well be that the effect of the host's diapause on the parasite can be communicated yet a step farther to a hyperparasite. Writing of *Brachymeria compsilurae*, which is hyperparasitic through *Compsilura concinnata*, Dowden (1935, p. 518) says of its hibernation, "This phase in the life history is confusing, for there does not seem to be any rule governing the diapause. In 1932 the number of larvae entering hibernation seemed to increase as the season advanced....In 1933, however, there seemed to be no definite hibernation tendency." But his data do show a very distinct tendency in complete agreement with those of 1932 with the single exception of one lot of puparia parasitized in June, and these may have been from a univoltine host. In view of the close parallel between Dowden's observations and those of Webber cited above, it seems likely that the diapause was transferred from the tachinid parasite to the chalcid hyperparasite.

Recent observations by Bradley & Arbuthnot (1938) on *Chelonus annulipes*, a braconid parasite of the corn-borer, provide an additional point. These authors find that *Chelonus* conforms to the life history of its host at two separate stages. Its larva remains in the first instar until the host is about to pupate. If the host matures steadily, there is no delay; but if the host is about to enter a diapause, the parasite larva remains undeveloped until the diapause is past and pupation of the host is imminent. As a result, in the summer generation of multivoltine *Pyrausta*, or in *Ephestia*, the parasite develops in less than two months; but in the winter generation of multivoltine *Pyrausta* or in the univoltine strain, in both of which a diapause is involved, development occupies ten months or a year respectively. In this part of its life history, *Chelonus annulipes* simply affords another instance of the phenomenon already described by Marchal in *Trichogramma cacaeciae* and it appears that here, as in Marchal's case, the condition of diapause of the host is the effector that inhibits the development of the parasite.

But *Chelonus* also conforms to its host at another stage where not the presence of an inhibitor but the absence of a stimulus seems to be the retarding factor. Bradley & Arbuthnot find, as Cox (1932, p. 16) had already observed in the related *Ascogaster carpocapsae*, that the egg of *Chelonus annulipes* develops step by step with that of its host. If the embryonic development of the host is delayed, so is that of the parasite; and if the host egg is infertile and therefore never hatches, that of the parasite similarly fails to develop. This need of a positive stimulus to development is quite different from the inhibitory factor studied by Marchal and is worthy of a closer investigation than it has yet received.

Not that this second point is unique. There are other examples in the literature in which hibernation and a diapause are not involved and where the influence of the host on the development of the parasite is a matter of days rather than of months. In some of these we can anticipate a similar explanation. According to Lathrop & Newton (1933, p. 153) the braconid *Opius melleus* remains in the first larval instar until its host, the apple maggot *Rhagoletis pomonella*, has formed its puparium. The variation of this period is not stated but must be considerable because the parasite attacks the host maggot in either its second or its third instar. Dowden (1934, p. 110) says that the tachinid, *Zenillia libatrix*, does not begin to develop rapidly until its host, the gypsy moth, pupates; so that there is a range of two weeks or longer in the time of development. Bess (1936, p. 603) reports a similar but less effect with another tachinid, *Leschenaultia exul*. Taylor (1937, p. 198) found that when the chalcid *Pleurotropis parvulus* was reared on the coconut beetle, *Promecotheca reichei*, the moulting of the host caused a slight delay in the development of the parasite. According to Morris (1937, p. 528) the egg of *Exenterus abruptorius* never hatches until its host, the sawfly *Diprion sertifer*, has spun its cocoon, a period ranging from 10 to over 30 days.

These, it will be noticed, are temporary effects elicited by rather specific activities of the host. Different from them are a few recorded observations in which there appears to be a correlation between the state of nourishment or health of the host and the rate of development of the parasite. Writing of *Chelonus texanus*, a braconid parasite of *Laphygma frugiperda*, Pierce & Holloway (1912, p. 428) remark that "the time of emergence of the parasite seems to depend almost entirely upon the size of the host. If it grows and feeds slowly, so does the parasite within." Tothill *et al.* (1930) observed a marked variation in the length of the larval period of *Ptychomyia remota*, a tachinid parasite of *Levuana iridescens* and consider (p. 188) that this variation "is not entirely a haphazard matter, but an adaptation to the condition of the host". They also write (p. 185) "any pathological condition arising in the host larva caused an abnormal, if adaptational, extension of the parasite's development". In *Pleurotropis parvulus*, a chalcid parasite of *Promecotheca nuciferae*, the rate of development varies with the stage of development of the host, according to Taylor (1937, p. 199), who attributes the effects to changes, as the host grows older, in the quality rather than the quantity of the food it provides for its parasites. Such correlations as these between the welfare of the host and the rate of development of its parasite may well turn out on further investigation to be rather common; but they must be carefully distinguished from the effect of environmental factors acting simultaneously on both parasite and host, mentioned at the beginning of this section.

That hosts may influence the rate of development of their parasites has now been amply illustrated. It remains only to point out that from the examples here cited, three different types of effectors appear to be concerned. (1) A specific physiological condition of the host, its state of diapause, is transferred to the parasite and inhibits its development. This may be assumed to take place through the medium of a chemical substance which is present in the host and is communicated to the parasite (*Trichogramma cacaeciae*, etc.). (2) Certain definite activities of the host, such as hatching or pupation, produce conditions affecting the ecdysis or other life processes of the parasite. This influence seems to be exerted through the production of physico-chemical conditions which stimulate or favour the development of the parasite (egg of *Chelonus*, etc.). (3) The general condition of the host with regard to feeding and health influences the rate at which its parasite is able to develop. Here the effect is less precise than in either of the other two types, and probably acts simply through the provision of more or less, better or poorer, nourishment for the parasite (*Ptychomyia*, etc.).

(2) OTHER PHYSIOLOGICAL EFFECTS

Apart from the effects on rate of development described above, practically no direct effects of the host on the physiology of its parasite have yet been described. The only case I have found is my own note (1940, p. 91) on a few individuals of *Trichogramma evanescens* emerged from eggs of *Sialis lutaria*. This host is usually unsuitable for *T. evanescens*, but occasionally permits the development of individuals which, although of large size and structurally normal, are feeble, short-lived, and barren. Such examples as this, which verges on the problem of host suitability, are of comparatively little interest. It is likely that more interesting physiological effects will be discovered when a deliberate search is made for the effects of the host on parasites other than *Trichogramma*.

VII. EFFECTS ON BEHAVIOUR

From the observations that are brought together below, it appears that the host may influence the behaviour of its parasite at three different stages—as a larva, as an emerging adult, and as an adult selecting hosts for its own progeny. It is likely, however, that I have overlooked many pertinent data; and it is probable, too, that other kinds of effects still await description.

The influence of the host can be discerned in the behaviour of a parasitoid larva observed by Voukassovitch (1924, p. 170). He noticed that the larva of *Pimpla examinator* may develop in either the caterpillar or the chrysalid of *Oenophthira pilleriana*, which lives in a webbing on or between the leaves of the vine. If it has developed at the expense of a caterpillar, the full-grown larva emerges from its host and spins a cocoon for itself in the webbing beside the host remains. If, however, it has developed in a chrysalid, the *Pimpla* larva pupates inside the pupal shell of the host without spinning a cocoon. In this case, then, whether the larval parasite shall emerge from its host or not, and whether it shall spin a cocoon or not, appears to be determined by the stage of the host attacked.

A slightly different example is provided by *Achrysochorella orientalis* which was studied by Taylor (1937, p. 166). This chalcid is a secondary parasite attacking either *Dimmockia* or *Pleurotropis*, which are themselves chalcid parasites of a coconut beetle. When it attacks *Dimmockia*, the hyperparasite is endophagous throughout its development. When it attacks *Pleurotropis parvulus* or *P. painei*, on the other hand, the hyperparasite is at first endophagous but later emerges from the remains of its original host and becomes an ectophagous predator on other host individuals. In this case the feeding behaviour of the larval parasite is determined by the species of host on which it develops.

Effects of the host on the emergence behaviour of its parasite are probably very common although they have not often been specifically described. A good example is that of *Oligosita utilis*, an egg-parasite of coconut leaf-mining beetles in Fiji. According to Taylor (1937, p. 61), when this parasite develops on *Promecotheca reichei* it emerges by biting its way through the wall of the egg capsule, a covering of triturated leaf-tissue placed over its egg by the female beetle; but when it develops on another species, *P. bicolor*, it emerges by biting in the opposite direction through the tissues of the leaf on which the host egg was laid.

The same observer says that the place of pupation varies according to the species and the stage of the host attacked by *Pleurotropis detrimentosus*. Summing up the situation, Taylor writes (1937, p. 163): "Thus, the pupa of *P. detrimentosus*, according to the species and the stage of the host, may be found unprotected in the mine, or enclosed within a pupal skin of *D. javanica*, or inside a Hispid larval or pupal skin but otherwise not enclosed, or enclosed within a pupal skin of *P. painei* or *P. parvulus* inside a Hispid skin." In this example, the host determines whether the emerging adult shall have to make its way out of one, two, or three separate coverings of different constitution and texture. Many such cases could be cited and, indeed, to be quite meticulous, one would have to admit that, since the spatial relations of every individual host are peculiar to it, every host imposes minor adjustments of behaviour on its parasite at least during the process of emergence from the host remains.

The principal interest in this section, however, lies in the effect of the host on the selective behaviour of its parasite—in the application to a parasite, that is, of the so-called host selection principle. This theory states that if a species is capable of feeding on two or more species of hosts, individuals will tend to oviposit upon the kind of host on which they themselves developed. Since it was formulated by Hopkins in 1916 with reference to a beetle feeding on different species of pine, the principle has been widely discussed and strongly supported but, strangely enough, almost entirely with reference to phytophagous forms.

Wheeler (1923, pp. 56–7) was apparently the first to apply the idea to entomophagous insects when he used it as an explanation of the predilection of solitary wasps for a particular species of prey; but until recently there have been no experimental data to support his suggestion.

Indeed, in at least one parasite, the principle does not seem to operate. Hase (1925, p. 186) allowed females of *Trichogramma evanescens* developed on different hosts the choice of several hosts to lay in, including individuals of the species from which they had themselves emerged. He reports: 'Trichogramma unterscheidslos die Eier der oben aufgeführten Formen ansticht. Oft genug war zu beobachten, das ein Weibchen z.B. erst ein Ephestia-Ei, dann ein Cimex-Ei und dann ein Galleria-Ei usw. anstach, trotzdem das ursprüngliche Wirtsei—Mamestra—daneben lag.' Using the same species of parasite, I have already shown (1935, p. 417) that a pure strain reared for sixty-three generations exclusively on eggs of *Sitotroga cerealella* did not develop a preference for its accustomed host over a strange one. From recent experiments I can now add that this strain of *Trichogramma evanescens* still chooses eggs of *Ephestia* or *Agrotis* in preference to those of *Sitotroga*, although it has now for more than 260 generations had experience of no host other than *S. cerealella*. It appears from Hase's experiments and my own, then, that the host selection principle does not operate with *Trichogramma evanescens*.

Data published by Proper (1931, p. 51) on the chalcid *Eupteromalus nidulans* ovipositing in larvae of the satin moth in their hibernation webs, and on larvae of *Apanteles melanoscelus* in their cocoons, indicate that this parasite also fails to show a preference for the host from which it was itself reared; but the study was designed for other ends and the data are insufficient for certainty on this point. Similarly, observations on the host selection of *Pimpla examinator* made by Jackson (1937, p. 87) bring the problem to mind but do not contribute definitely to its solution.

The positive application of the principle to a parasitoid has, however, recently been demonstrated by Thorpe & Jones (1937) using the ichneumonid *Nemeritis canescens*. This species normally develops as an endoparasite of the larvae of the flour moth, *Ephestia*, but it can also be reared in larvae of the small wax moth, *Meliphora grisella*, if the adult parasite can be induced to attack that host. This can be brought about by putting *Meliphora* larvae into close association with *Ephestia* larvae so that they are contaminated with the smell of *Ephestia*.

Using an olfactometer technique, Thorpe and Jones first showed that adults of *Nemeritis* reared from *Ephestia* are strongly attracted to the smell of *Ephestia* larvae, and that they show no reaction whatever to the smell of *Meliphora*. Parasites reared from *Meliphora*, on the other hand, while still preferring *Ephestia* if given a choice of the two hosts, nevertheless show a slight decrease in the intensity of their response to *Ephestia* and a marked tendency to respond to the smell of *Meliphora*, whether they encounter it alone or in competition with that of *Ephestia*.

Seeking the cause of this change of behaviour, Thorpe and Jones found that part of it is due to a conditioning of the young adult parasites regardless of their hosts, since adults actually reared from *Ephestia* but conditioned by contact with *Meliphora* larvae immediately after their emergence, are different in their olfactory responses from adults not so conditioned (Table VII). But not all of the altered behaviour is due to such exposure and at least some of the effect results from the influence of the host during the pre-imaginal period. It is, of course, this latter point that is of particular interest to us here, because it amounts to a demonstration that the host has affected the selective behaviour of its parasite.

Table VII. *Olfactory responses of* Nemeritis canescens
(*data from Thorpe & Jones*, 1937)

Parasites	Choice of		Choice of		Choice of	
	Ephestia	Blank	*Meliphora*	Blank	*Ephestia*	*Meliphora*
Reared on *Ephestia*	87·3	12·7	50·8	49·2	85	15
Reared on *Ephestia* but "conditioned" to *Meliphora*	—	—	61·6	38·4	83·4	16·6
Reared on *Meliphora*	84·4	15·6	66	34	65·8	34·2

The case of *Nemeritis*, however, does not provide exactly the kind of example one might wish because the parasites reared on *Meliphora*, although stimulated by the smell of that species, still show a marked preference for *Ephestia*. An instance in which the parasite actually chooses its own host species in preference to the ancestral host has yet to be described among entomophagous forms. Parasites that have been introduced into new areas for the biological control of pests related to but different from their original hosts would provide favourable material for this experiment, which would be of the greatest interest in the general problem of the segregation and divergence of new forms.

VIII. CONCLUSIONS

The principal result of this review emerges clearly from the foregoing pages. It is evident that, far from being a purely passive victim, obliterated without a trace, the host is often able to impress its mark, and a very clear mark at that, upon the insect parasitoid that destroys it. Proof is already available of effects of the host on the size, form, rate of development, and behaviour of its parasite; evidence can be found of influence on fecundity, longevity and vigour; and it is likely that many other effects still await discovery and description. There can be no doubt that the host may bequeath to its parasite an important and sometimes striking legacy of morphological, physiological, and behaviouristic characters.

It follows that the host is one of the important factors that must be considered and controlled in any critical, and especially any quantitative, study of insect parasitoids. Accounts of behaviour, data on rate of reproduction and longevity, measurements of length, even descriptions of structure—none of these have any absolute validity for the species, none of them can be considered "biological constants" of a parasitoid, unless the host is known and recorded. This is perhaps the most generally applicable result of the review: the demonstration of the need in insect parasitology to rear parasites from known and standardized hosts, as well as under controlled environmental conditions, if data of general value are to be obtained.

Apart from these general conclusions, several more particular matters emerge from the review, of which two deserve special mention. The diapause in insects has been accounted for by two main hypotheses: the temporary absence of a factor necessary to maintain growth; and the presence of a chemical inhibitor of development. The second of these receives support from facts that are here brought together. The power of the host to transfer a state of diapause from itself to its parasite has been illustrated above by several examples. In at least one of those examples, that of Marchal's *Trichogramma cacaeciae*, and possibly in others, the host is completely destroyed and ingested before the diapause overcomes the parasite. In this case, therefore, the only way in which the host can produce its effect is as an article of food. No other food is taken by the parasite, so that a factor lacking in the host when it is eaten cannot be supplied later. The conclusion seems inescapable that here a chemical inhibitor is responsible for the diapause of the host and that it is ingested by and then acts upon the parasite.

The second matter is scarcely a conclusion so much as the uncovering of a further problem. In the case of *T. semblidis* the interest now lies not so much in the influence of the host on the dimorphism of the parasite as in the extraordinary power of the parasite to keep its alternative form of male hidden but latent. We have seen that throughout sixty-four successive generations only one form of the parasite was regularly produced and that in the 65th generation, when a particular species of host was provided, the other form suddenly and almost exclusively appeared. Where was this alternative form during those sixty-four generations? If the explanation is offered that the species has in every generation two potentialities, one of which is elicited by one host and one by the other, we must still ask whether in this species, and in others, there may not be other still unknown forms which will remain latent until they are brought to light by the efficient host. And if that is so, what becomes of our concept of specificity of organization, if different stimuli and environments can so strikingly and fundamentally change it?

The disproportionate reference to species of *Trichogramma* in this review should not be too lightly attributed to my special interest in the genus or to my better acquaintance with its literature. It is a fact that, at the moment, many of the best data on the effects of hosts on parasites refer to *Trichogramma*: to *T. evanescens* for effects on size; to *T. semblidis* for the most striking effect, on structure; and to *T. cacaeciae* for the effects of hosts in diapause. In my opinion, this is not altogether due to peculiarities of this genus, but to the fact that, owing to their ready acceptance of a laboratory existence, the species of *Trichogramma* have been more continuously reared and studied than other parasitic insects. Some, at least, of the effects of hosts on their parasites are rather subtle; and it is only when the norm has been ascertained by long-continued and controlled rearing upon a variety of hosts that the vagaries of form and function impressed upon the parasite by its host become evident.

IX. SUMMARY

1. The size of many insect parasitoids is influenced by the size of the different species and individuals of hosts on which they develop.
2. Through its effect on gross size, the host may influence the size and proportion of parts, the presence or absence of wings, the developmental period, the fecundity, the vigour, and the behaviour of its insect parasite.
3. One case is known in which the fundamental dimorphism of an insect parasite is controlled by the species of host on which it develops.
4. The rate of development of many insect parasites is influenced by the host. In the most striking cases, the transfer of a state of diapause from the host to the parasite is involved, and the host may determine how many generations the parasite shall have in a year.
5. Different species of hosts may cause individuals of a parasitic species to behave differently. The behaviour of the larval parasite may be affected, or that of the emerging adult, or that of the adult parasite seeking hosts for its own offspring.
6. In view of these various effects of the host on the morphology, physiology, and behaviour of its parasite, it is necessary to control and standardize the hosts used for any precise work in insect parasitology.

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PREDACEOUS FUNGI

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I. THE PREDACEOUS SERIES OF HYPHOMYCETES

THE capture of actively motile animals by a fungus was first made known by Zopf (1888) a little more than half a century ago, in an account setting forth the destruction of nematodes by *Arthrobotrys oligospora* Fres. Apparently even at that time this hyphomycetous form was not unfamiliar to mycologists generally. Its handsome and easily noticeable conidial apparatus, which develops readily on many kinds of decaying vegetable material and animal excrement after incubation in a damp chamber, had been well figured and described by Fresenius (1852) in his original characterization of the species. To illustrate the formation of successive conidial heads, some of Fresenius's figures had been copied in both the first and the second edition of de Bary's (1866, 1884) widely used manual. Münter (1866), Loew (1867, 1874), Karsten (1867), and Harz (1871) had made the fungus a subject of discussion in a controversy concerning its supposed identity with *Trichothecium roseum* Link. De Bary (1867) likewise had contributed to the discussion in pointing out the marked difference between the two species with such clarity that their continued confusion during later periods might have been spared. Woronin (1870), after expressing himself in full support of de Bary's view, described conidial germination in *Arthrobotrys oligospora*. The conidium when placed in moist soil, in fresh manure, or in water, was sometimes found to give rise only to an ordinary branched septate mycelium; but at other times, under similar external conditions and therefore without any evident external cause for change in behaviour, the growing germ hypha would recurve so as to bring its tip in contact with the spore and the juxtaposed parts would anastomose, thereby bringing about the formation of a curious bail-like structure. One or several recurving branches would then be put forth from this structure with consequent development of additional bails. Repetition of the process, often varied without apparent cause by production of undifferentiated hyphae, and frequently modified besides by fusion between individual bails as well as between bails and undifferentiated hyphae, resulted in the production of a somewhat extensive irregular hyphal network, which Woronin always observed to be prostrate on the surface of the substratum.

The hyphal bails and networks which Woronin made known, presumably because of their morphological oddity, were found by Zopf to have the remarkable function of capturing motile eelworms. Capture was held to take place wholly as a result of mechanical entanglement initiated when the animal thrust itself, often rather energetically, into the opening of a bail-like part, or into a number of such openings. Failure of the eelworms to extricate themselves in spite of their prolonged struggles was attributed by Zopf to the elasticity of the turgid hyphal elements, whereby supposedly these elements were enabled to retain a clinging hold on a captive, much as a loop of rubber tubing clings to a finger thrust into it.

Zopf used Geissler chambers for direct continuous microscopic observation on the mode of capture and on the details of invasion. After vegetative development of *A. oligospora* had been well started within these chambers in clear diluted juice of sweet cherries, Zopf introduced into the individual culture a drop of water containing active larvae of the wheat-cockle nematode, *Anguillulina tritici* (Steinbuch) Gervais & v. Beneden. Repeatedly he observed eelworms that had entered into hyphal bails being held fast, and, after violent struggles, becoming motionless; the periods of time required in two instances for an inert condition to supervene were recorded as 2 and 2½ hr., respectively. The fungus, immediately after boring through the cuticle of a captive, intruded two or three bud-like cells between musculature and integument, indenting the musculature and separating it more or less extensively from the integument. One or more lateral branches then grew out from the intruded process and extended themselves between the internal organs, their continued elongation bringing about paralysis and death of the captive, and conspicuous fatty degeneration of all its fleshy structures. Eventually the fatty material, but not the cuticle and spicule, was completely assimilated by the fungus and utilized for the production of new branches, which broke through the integument to form new systems of hyphal bails outside.

Zopf's employment of Geissler chambers, wherein capture of nematodes and expropriation of their contents could be observed microscopically, was undertaken after he had noticed that eelworms were consistently present in all his cultures of naturally decaying materials (such as partly submerged algal masses, horse dung, and rabbit dung) on which *Arthrobotrys oligospora* was found developing spontaneously, and that a large proportion of the eelworms were always dead, their bodies evidently permeated with hyphae traceable to mycelia of the fungus. As he found, moreover, that under natural conditions dung substrata of *A. oligospora* frequently contain eelworms permeated with hyphae of the fungus, he concluded that in nature capture of nematodes proceeds in exactly the same manner that he had found in his cultures. In addition to the wheat-cockle parasite, he observed destruction of various unidentified coprophilous, saprophilous and aquatic eelworms belonging to different genera.

Two years after the appearance of his paper on nematode parasites, Zopf (1890) published his meritorious yet apparently little used treatise on fungi, wherein he devoted several passages of some length to his discoveries concerning *A. oligospora*. In one of these passages the specialized predaceous apparatus of *A. oligospora* was set forth as the only example then known of a loop mycelium, a type of vegetative structure co-ordinate with six other vegetative types, namely: (1) typical filamentous mycelium; (2) yeast-like, budding mycelium; (3) haustoria, appressoria and climbing organs; (4) sclerotia; (5) mycelial strands, mycelial plates, and rhizomorphs; and (6) reduced mycelia, represented especially in the thalli of the Chytridiales and Ancylistales. An adequate summary of Zopf's findings was likewise presented by Ludwig (1892) in a text-book dealing especially with the bacteria, fungi and algae of significance to man or playing an important role in the economy of nature. In this summary *A. oligospora* is designated as a fungus with snares, and is placed in a category with members of such insectivorous phanerogamic genera as *Drosera*, *Utricularia*, *Pinguicula*, *Aldrovanda* and *Dionaea*.

Notwithstanding Ludwig's discerning analogy, the distinctiveness of the biological relationship between *Arthrobotrys oligospora* and nematodes was apparently rather slow in gaining recognition among mycologists. Matruchot (1892), in a comparative study devoted partly to the morphology and taxonomy of the fungus in question, made incidental reference to Zopf's work on "un *Arthrobotrys oligospora* vivant en parasite sur une anguillule (Tylenchus)", without indicating that the parasitism concerned was of an unusual kind. Guéguen (1904) included a brief account of *A. oligospora* in his synoptic treatise on the fungi parasitizing man and animals. In this account he stated rather cautiously that according to Zopf it would seem the fungus incloses eelworms in the coils of its mycelium and grows into the bodies of these animals, though perhaps a little more confidence might be held to underlie his use of the term "anastomoses-pièges" for hyphal bails shown in the meagre but well-chosen assortment of relevant figures which he copied after Zopf. Coupin (1909), in his atlas of fungi parasitic and pathogenic on man and on animals, included a few figures of *A. oligospora*, with comments to the effect that the fungus, while generally living saprophytically, also attacks eelworms. The same comment was applied to *A. superba* Corda, although that species could at the time have been considered as implicated in destruction of nematodes only on the ground of its supposed identity with *A. oligospora*.

A better foundation for an understanding of the true import of Zopf's findings was provided after a lapse of 23 years when Sommerstorff (1911) reported his observations on the capture and destruction of living rotifers by an aquatic phycomycete which he discovered and named *Zoophagus insidians*. Sommerstorff, in his extended original account, compared the habits of this phycomycete to those of the Venus' fly-trap, and pointed out appropriately that the only other fungus then known to exemplify a similar mode of life was *Arthrobotrys oligospora*, which species, however, he considered to be pronouncedly saprophytic. Later, in comparing *A. oligospora* with *Zoophagus insidians*, he (1912) intimated that the former had recourse to capturing eelworms only in case of necessity, when other nourishment was insufficient, whereas the latter was from the beginning adapted for capture of animals. Mirande (1920), who studied *Z. insidians* in France, again cited *Arthrobotrys oligospora* as the only other fungus capable of capturing living animals. Gicklhorn (1922), in reporting observations on the same phycomycete in Austria and Jugoslavia, likewise cited the analogy offered by *A. oligospora,* with the comment that while the latter fungus could without untoward effects obtain its nourishment in a wholly saprophytic manner, *Zoophagus insidians* became depauperate when developing as a facultative saprophyte. Pfeiffer (1927–8), in reviewing the literature on carnivorous fungi and liverworts, set forth *Arthrobotrys oligospora* as being much more capable of subsisting saprophytically than either *Zoophagus insidians* or *Sommerstorffia spinosa.*

Studies by Rahm (1922) on the fauna of mosses and lichens, carried out on material originating largely in Germany, gave occasion for the first published report confirming capture of nematodes by *Arthrobotrys oligospora.* Species of *Tylenchus* and *Mononchus* were observed to be attacked especially often. The portion of predaceous mycelium figured by Rahm shows much closer septation in the bail-like parts, as well as in the unmodified filamentous parts, than is usual in *Arthrobotrys oligospora.* In cellular make-up the hyphal loops appear little like those illustrated earlier by Woronin and by Zopf; and the comment that an enveloping loop winds closer and closer about the captive seems hardly applicable to the predaceous apparatus of *A. oligospora.*

Korab's (1929) citation of *A. oligospora* among the fungi he found destructive to the cysts of the sugarbeet nematode *Heterodera schachtii* Schmidt in Russia presumably implies ordinary parasitism on relatively inert stages of the animal rather than capture and destruction of actively motile individuals. On recording *Arthrobotrys oligospora* (under the synonym *A. superba* var. *oligospora* (Fresen.) Coemans) as being common in dung cultures prepared in Winnepeg, the statement is added by Bisby *et al.* (1938) that it was observed by Buller to form mycelial loops wherein larval nematodes were snared and killed.

It is not certain whether actual observations on predaceous activity can safely be read into a passage by Vañha (1911) characterizing the fungus "*Trichothecium* (Link)" as one which captures and destroys nematodes in the soil. Disregarding this doubtful passage and a number of publications wherein destruction of nematodes by *Arthrobotrys oligospora* receives attention at secondhand, including, for example, writings by Sartory (1920–3), Kostka (1928), Goffart (1932), and Stehli (1934), it would appear that during the half-century following Zopf's investigations, observation on natural substrata have yielded only two confirmatory records. Owing to their opaqueness, natural solid substrata offer very obvious difficulties for the study of predaceous activity in terricolous fungi; for while the fairly tall conidiophores of some of these fungi are easily seen, the predaceous apparatus is nearly always buried in the substratum and is thus rather effectually concealed from view. Such optical difficulties may conveniently be obviated by using transparent gel culture-media that encourage liberal multiplication of suitable microscopic animals, or at least permit their maintenance in a living condition for extended periods.

The superior merits of transparent preparations were exploited by Drechsler (1933*a*, 1933*c*, 1933*d*, 1934*a*, 1937*b*, 1940*b*) in his utilization of maizemeal plate cultures started from diseased rootlets or from other decaying plant materials. In large part the plate cultures were originally prepared for the isolation of oomycetous parasites, including especially root-rotting species of *Pythium*, *Phytophthora*, *Aphanomyces* and *Plectospira*. As the oomycetous mycelia growing out from the decaying plant tissues encouraged fairly abundant development of bacteria, the cultures afforded free multiplication of nematodes habitually feeding on bacterial slime as well as of nematodes that subsist by sucking the contents of other nematodes or of fungus hyphae. Often additional putrescent material was then introduced by placing two or three pinches of friable leaf mould firmly on the surface of the agar. After the eelworms had attained large numbers, fungi subsisting on them would begin growing out from the deposits of decaying material and would then extend their mycelia widely through the infested culture. This extension of vegetative hyphae and the concomitant formation of predaceous apparatus resulted in widespread capture of nematodes. By mounting portions of substratum on a microscope slide, the details of the capture and expropriation of prey could be conveniently studied under objectives of high magnification.

In the cultures thus prepared and utilized there were brought to light, besides *Arthrobotrys oligospora*, twenty other nematode-capturing hyphomycetes apparently related to it. Of these twenty forms, three produced asexual reproductive apparatus whereby they could be referred with moderate though not complete certitude to species known under the binomials *A. superba* Corda, *Dactylella ellipsospora* Grove, and *Dactylaria candida* (Nees) Sacc. The remaining seventeen fungi were described by Drechsler as new species under the following binomials: *Arthrobotrys cladodes*, *A. conoides*, *A. musiformis*, *A. dactyloides*, *Dactylella bembicodes*, *D. asthenopaga*, *D. lysipaga*, *D. leptospora*, *D. gephyropaga*, *D. doedycoides*, *Dactylaria brochopaga*, *D. thaumasia*, *D. polycephala*, *D. haptospora*, *Triposporina aphanopaga*, *Trichothecium polybrochum*, and *Tridentaria implicans*. As may be inferred from their assignment to six separate genera in the Mucedinaceae, the twenty-one nematode-capturing hyphomycetes reveal considerable diversity with respect to morphology of conidiophore and conidium. They also reveal, in some measure uncorrelated with the features governing demarcation of genera, equally notable diversity with respect to morphology and operation of predaceous apparatus.

Essentially the same type of predaceous apparatus as that described for *Arthrobotrys oligospora* by Woronin and Zopf is produced not only in four congeneric forms, namely *A. superba*, *A. cladodes*, *A. conoides*, and *A. musiformis*, but also in two species of *Dactylaria*, namely, *D. thaumasia* and *D. polycephala*. When the hyphal bails and networks of these fungi are observed operating in a relatively moist agar medium, eelworms may often be seen securely held even though they are only loosely surrounded by an anastomosing element, or, again, even though they are in contact only with the periphery of such an element. Indeed, even an unmodified portion of filament in close mycelial connexion with a bail or network may sometimes be seen holding fast a struggling nematode. Careful examination shows that in such instances of capture the animal is held fast exclusively by means of a colourless, hyaline, adhesive material, which the fungus apparently secretes locally. Further inspection reveals that even when the nematode is enmeshed in one or several hyphal bails, the same kind of adhesive material is present wherever the struggling prey remains in persistent contact with the fungus. Therefore capture is not, as Zopf believed, a matter wholly of structural engagement, but is accomplished either through adhesion alone, or through adhesion together with structural engagement. On agar substrata moist with free liquid water and bacterial slime, nematodes are able to exert little effective traction, with the result that they are caught in large numbers merely through adhesion. However, on fairly dry agar, where more effective leverage is possible, only the smaller and feebler eelworms are captured solely through adhesion, the larger and more robust specimens being brought to submission only when their struggles are hampered through encirclement in one or several hyphal meshes.

Zopf's failure to see adhesive material participating in the operation of predaceous structures in *Arthrobotrys oligospora* must be attributed to the shortcomings of the microscopes available in his time. To optical difficulties may perhaps be attributed likewise his rather unhappy description of the initial stages in the invasion of captured eelworms. Following narrow penetration of the cuticle, extension of an infective process circularly between integument and musculature is much less frequent than is intrusion of a globose body that increases in size until in an hour or two it occupies the animal's transverse section completely or nearly completely. Sometimes, indeed, a globose body may locally distend the integument beyond its normal compass. Though unmistakable examples of these intrusive structures were shown in one of Zopf's (1888, Tab. XVII, fig. 9) figures, he made no comment concerning them beyond the mere statement that penetration of the fungus had occurred. Yet there appears ample reason for distinguishing the globose body as a special structure. For by virtually severing the animal internally it would seem to serve a very distinctive function in reducing the captive to an advanced state of disablement, so that filamentous haustorial hyphae may without much delay or much danger of mechanical injury be extended to appropriate the fleshy contents.

While *Dactylella gephyropaga* also produces an adhesive network for capturing nematodes, it produces its predaceous apparatus in a manner not known among other retiary species. From filamentous mycelial hyphae a series of columnar processes is put forth nearly at right angles and at intervals often approximating to 25μ. After these processes have attained a length of about 20μ, they abruptly alter their direction of growth; further elongation taking place along an axis parallel to the parent filament and toward the tip of a neighbouring process. When this tip has been reached, as also when the prolongations from two neighbouring processes meet midway, anastomosis takes place, with resultant formation of a rectangular mesh, which may remain discrete or may become united with other meshes into a scalariform network. Further development may superimpose secondary processes and meshes, which, however, usually lack the geometrical orientation often characterizing homologous primary units. In any case, whether they be of primary or of secondary order, the columnar processes, from an early stage in their development, are capable of capturing eelworms through secretion of hyaline adhesive material. After the completion of rectangular meshes, adhesion is made more effective owing to the restraint on struggling prey imposed through hyphal engagement, so that somewhat larger and more energetic animals become subject to capture. Penetration and invasion of the prey proceeds as in the seven retiary species with curved predaceous elements: the animal's integument is narrowly perforated, its body is severed internally through intrusion of a globose structure, and assimulative hyphae are extended lengthwise through its fleshy interior.

If a predaceous process of *D. gephyropaga* were arrested in its development on attaining a uniseptate columnar stage, if its basal cell or basal portion were reduced to a non-adhesive stalk having about the same width as the parent filament, and if, further, the distal adhesive cell were modified into a globose knob about three times as wide as the supporting stalk, the resulting structure would correspond to the predaceous organ which enables three species, *D. ellipsospora*, *D. asthenopaga*, and *Dactylaria haptospora*, to capture nematodes. This type of predaceous organ was first described and figured by Zopf (1888) in his very short account of a fungus he found destroying a conspicuously small, slender eelworm in rabbit dung. However, possibly because true conidiophores and conidia were lacking in his material, or possibly because on the opaque substratum their connexion with the vegetative mycelium was concealed, he interpreted the globose cells as conidia and the short stalks supporting them as conidiophores. Zopf noted the application of the supposed conidia to eelworms, but made no mention of the adhesive secretion whereby the animals are prevented from bringing such application to an end. Presumably the microscopes of his time did not reveal the adhesive secretion, and therefore not only prevented him from recognizing the knob-bearing fungus as a second nematode-capturing species, but also made him miss a clue that very probably would have led him to a more complete explanation of the manner in which the hyphal bails of *Arthrobotrys oligospora* operate in capturing prey.

The bodies that Zopf took for conidia and that presumably suggested the generic term in the binomial *Monosporidium repens*, which he applied to the fungus on rabbit dung, correspond very closely to the stalked adhesive knobs in a nematode-capturing form whose conidia and conidiophores agree rather well morphologically with conidial apparatus described by Grove (1886) two years earlier as *Dactylella ellipsospora*. Grove's description was drawn from material found on rotten wood in England, but apparently conidial apparatus of the same species was later observed by Bubák (1906) on rabbit dung in Bohemia, and was redescribed by him under the name *Monacrosporium leporinum*. It must be admitted that application of Grove's binomial to the knobbed nematode-capturing fungus with the solitary, four-septate, broadly fusiform conidia is not as free of uncertainty as might be desired. Thus, some of the narrower specimens among the four-septate conidia of *Dactylella gephyropaga* also conform rather well to the description given of the spores of *D. ellipsospora*. Further, while the adhesive knobs of *D. asthenopaga* and *D. haptospora* are for the most part somewhat smaller in their largest dimension than 8–10μ, and consequently conform less well to Zopf's description than the knobs produced by the fungus referred to Grove's species, the differences in size are not pronounced.

Since the stalked knobs hold prey merely through adhesion unaccompanied by structual engagement, the three species provided only with predaceous organs of this type depend mainly on capture of the smaller and feebler eelworms. On fairly dry agar substrata *D. asthenopaga* is often limited to the destruction of the relatively sluggish nematodes belonging in the genus *Bunonema*, though on moister agar media, where the animals cannot exert equally effective leverage, it also captures specimens of more active genera. After capture is effected the knob-bearing forms, much like the retiary species, narrowly perforate the animal's integument, intrude a massive bulb, and extend assimilative hyphae lengthwise through the fleshy interior. Zopf accurately described and figured the manner in which his *Monosporidium repens* invaded the eelworms it attacked, thereby supplying a happy contrast to the less satisfactory treatment of this phase in his account of *Arthrobotrys oligospora*. The globose body he appropriately designated as a short, wide infection bladder, though apparently without surmising its function in subduing unwilling prey.

Adhesive knobs of smaller size are produced by *Dactylella lysipaga*, *D. leptospora*, and *Dactylaria candida* on slender, longish, lateral stalks. In none of the three forms mentioned, however, do these smaller knobs comprise the only type of predaceous apparatus, since, in addition, all three give rise, likewise on longish narrow stalks, to delicate, mostly triseptate hyphal rings. Eelworms that stray into these rings are soon tightly encircled, though very often the frail stalk breaks, permitting the animal to continue on its way. Penetration by the fungus is usually somewhat tardy, and the pouch-like part first intruded into the animal is not usually large enough to bring about prompt disablement. Accordingly, nematodes may become encumbered by several rings before their diminishing movements permit extension of assimilative hyphae. Whether adhesive material is present on the rings remains uncertain. Ordinarily the delicate annular structures are jammed on to the nematode so firmly that their persistence can readily be attributed to physical engagement alone. Active constriction has never been observed in rings of the type here concerned, their snug encirclement of eelworms apparently being brought about wholly by the forward movement of the animals themselves.

A fungus which captures nematodes by means of non-constricting rings apparently wholly similar to those of *Dactylella lysipaga*, *D. leptospora* and *Dactylaria candida* was found by Sherbakoff (1933) to be associated with strawberry roots in Tennessee. As this investigator evidently observed no connexion with aerial conidiophores and conidia of familiar type, he held the rings themselves to constitute asexual spores of a new type. To make taxonomic provision for reproductive apparatus of such presumptive novelty, he erected a new genus *Anulosporium*, and assigned his fungus, under the name *A. nematogenum*, to the Mucedinaceae-Helicosporae. Unsuccessful in his attempts to grow the predaceous organism on agar media, Sherbakoff encountered the optical difficulties inherent in opaque natural substrata, and in consequence was led, much like Zopf before him, to a serious yet understandable misinterpretation and to an erroneous taxonomic disposition. Incidentally, Sherbakoff held the stalked knobs produced by his fungus to represent an early stage in the development of the rings. Observations on *Dactylella lysipaga* and *Dactylaria candida* growing on transparent media indicate, however, that the non-constricting rings of these species do not ordinarily pause at an early knob-like stage, but continue developing without interruption to their definitive condition. In *Dactylella leptospora*, where adhesive knobs, though frequent on detached conidia, are only rarely found arising from mycelial hyphae side by side with the rings, the sequence of development suggested by Sherbakoff seems especially improbable.

Annular predaceous organs which like the non-constricting loops just considered are regularly composed of three arcuate cells, but which differ from these loops in being of much sturdier construction as well as in being attached much more securely by a short, stout, two-celled stalk, operate both to capture and to disable eelworms in cultures of *Arthrobotrys dactyloides*, *Dactylella bembicodes*, *D. doedycoides*, *Dactylaria brochopaga*, and *Trichothecium polybrochum*. When formed in agar media these sturdier rings are for the most part oriented approximately at right angles to the parent filament, and frequently are spaced at more or less regular intervals. Entrance of an eelworm into the aperture of any one of these structures causes the three arcuate cells to contract abruptly with pronounced centripetal swelling. Through the violent closure thus effected the animal is not only prevented from escaping but is constricted so severely that its disablement soon follows, permitting extension of assimilative filaments lengthwise within its fleshy body.

The nature of the stimulus causing closure of the constricting ring has been investigated by Couch (1937), who worked with *Dactylella bembicodes*, and also by Comandon & de Fonbrune (1938, 1939), who used *Dactylaria brochopaga* and *Dactylella bembicodes*. Employing a micromanipulator, Couch observed only slight swelling when a glass rod was moved on the inner surface of an open ring, and concluded that mechanical irritation plays a very small, if any, part in closure of the loops. Application of heat, whether in a hot scalpel or in water at a temperature of 33–75° C., he found to be consistently effective in springing the traps, even though, as he recognized, a thermal stimulus cannot well be postulated in the normal entrapment of 'cold-blooded' animals. On the other hand, Comandon & de Fonbrune found that closure could be induced either by thrusting a living nematode into the orifice of an open ring, or by gently rubbing the inner surface of any one of the three constituent cells with a microneedle. Pronounced centripetal dilation of the cell stimulated by friction was recorded by means of motion pictures and in this way found to ensue in the course of 0·1 sec.; the other two cells as a rule reacted similarly a fraction of a second later. Whatever may be the stimulus bringing about closure, it seems certain that the pronounced constriction exerted on captured eelworms makes adhesion wholly unnecessary for retention of the prey within the strangulating loops. Accordingly, Drechsler (1940*b*), on observing, in a culture of *D. doedycoides*, a nematode tethered by a very elastic band to the lateral surface of a ring which had closed while empty, was inclined to the view that the adhesive material of which the band was composed more probably had its normal function in helping to spring the trap than in helping to hold the captive.

The two remaining nematode-capturing hyphomycetes, *Tridentaria implicans* and *Triposporina aphanopaga*, apparently do not produce predaceous parts sufficiently differentiated to be distinguished with any certainty before prey is engaged by them. Neither species has been observed to consume any but feeble eelworms, which are apparently held by adhesion to tips of hyphal branches. *T. aphanopaga* has been found subsisting mainly on small larvae of *Plectus parvus* Bastian and *P. communis* Bütschli, while *Tridentaria implicans* has so far only been seen living on specimens of *Aphelenchoides parietinus* (Bastian) Steiner that had already been much enfeebled from extensive invasion by a minute protozoan parasite. The globose structure initially intruded by either fungus is often no wider or only slightly wider than the external portion of the infective branch, especially after this branch has become noticeably stouter with the accession of food materials from the assimilative hyphae extended lengthwise through the animal. In *Tridentaria implicans* a curious development takes place subsequent to capture, in that the infective branch grows out laterally just below its adhering tip to encircle the nematode after a manner frequently suggestive of encirclement by a non-constricting ring.

Drechsler (1934*b*, 1935*d*, 1936*c*, 1937*c*, 1939*c*, 1940*a*) has described as new, under the binomials *Tridentaria carnivora*, *Pedilospora dactylopaga*, *Dactylella tylopaga*, and *D. passalopaga*, four hyphomycetous species that subsist habitually by the capture of terricolous rhizopods. Owing to the lesser activity of the animals caught by them, the four species would hardly be expected to present as highly specialized predaceous apparatus as is formed in the generality of nematode-capturing forms. And, indeed, one of the species, *Tridentaria carnivora*, like the nematode-destroying form assigned to the same genus, appears to be lacking in distinctive predaceous organs. Apparently it captures its prey, the testaceous rhizopod *Difflugia constricta* (Ehrenb.) Leidy, by causing the exposed portion of the animal's sarcode to adhere to outwardly undifferentiated mycelial hyphae whereupon it extends a branching haustorial system into the interior of the shell to assimilate the protoplasmic contents.

Pedilospora dactylopaga is known to prey on four species of shelled rhizopods: *Difflugia globulosa* Duj., *Trinema enchelys* Ehrenb., *Sphenoderia dentata* Penard, and *Geococcus vulgaris* Francé. At rather regular intervals on its hyphae there are borne distinctive digitate protuberances, which hold fast to susceptible animals by means of their adhesive tips, and then grow into them as more or less branched haustorial prolongations. Though measuring usually only about 2·5–5μ in length and about 1·2–1·5μ in width, the digitate protuberances of *Pedilospora dactylopaga* invite comparison more especially with the columnar adhesive processes of *Dactylella gephyropaga* before their initiation of bridging connexions.

Globose or prolate ellipsoidal adhesive protuberances on the mycelial hyphae of *Dactylella tylopaga* serve that fungus in the capture of *Amoeba verrucosa* Ehrenb. After the thick pellicle of an adhering animal has been perforated, the protuberance grows into the massive sarcode as a stout hyphal prolongation, which on repeated bifurcation gives rise to a closely dichotomous haustorial system. As a rule the haustorial system is at first continuous or sparingly septate, but later it becomes divided into many cells, some of which put forth filamentous hyphae that pass through the collapsed pellicle to extend the predaceous mycelium farther over the substratum.

Dactylella passalopaga is known to capture two species of shelled rhizopods, *Geococcus vulgaris* and *Euglypha laevis* Perty. When occasion offers both these protozoans are given to feeding on fungus mycelium, which they accomplish by applying their relatively small mouths to hyphae. In response to such contact the hypha of *Dactylella passalopaga* burgeons forth a lateral process into the animal's mouth. Once inside, the process expands at its apex to a width greater than the width of the oral opening, so that the rhizopod can no longer disengage itself. With specimens of *Geococcus vulgaris* a simple terminal swelling is usually sufficient to prevent escape; whereas specimens of *Euglypha laevis*, because of their somewhat larger mouths, require a more massive gag consisting usually of several lobes. From the intruded gag, whether simple or compound, one or several assimilative hyphae are extended into the sarcode to appropriate the protoplasmic contents.

II. THE PREDACEOUS ZOOPAGACEAE

Observations on agar plate cultures started from diseased roots or from other plant materials found decaying in moist contact with the soil, have led to the description by Drechsler (1933*a*, 1933*b*, 1933*c*, 1934*a*, 1935*a*, 1935*b*, 1935*c*, 1936*a*, 1936*b*, 1937*a*, 1938, 1939*a*, 1939*b*) of twenty-four mainly terricolous fungi, which, like the twenty-five hyphomycetes already discussed, subsist habitually through capture of animals, yet which are taxonomically alien to the mucedinous series. In these twenty-four species the filamentous hyphae that make up the vegetative or predaceous mycelium are at the beginning devoid of septa, though in later stages a retaining cross-wall may come to delimit living portions from degenerating or evacuated portions, and though, again, partitions normally appear in connexion with sexual and asexual reproduction. Their mycelial structure therefore clearly marks these fungi as phycomycetes, while their initiation of sexual reproduction, wherever this phase of development is known, through union of outwardly similar hyphal parts, characterizes them as zygomycetes. Very often the ripe sexual spore resembles that of many oomycetes in showing a homogeneous central reserve globule within a parietal granular layer, and in some species, besides, the zygosporangial membrane only loosely surrounds the sexual spore proper much as the oogonial envelope in many species of *Pythium* and *Aphanomyces* loosely surrounds the single oospore.

In their asexual reproduction the predaceous phycomycetes give rise to conidia of various distinctive shapes, ranging from filiform to subspherical. Though several among them form their spores in chains, none show any evidence of endogenous conidial development comparable to the development of seriate spores in the genera *Piptocephalis*, *Syncephalis* and *Syncephalastrum*. None, again, show the least structural modification for the forcible discharge of conidia after the manner usual among the Entomophthorales. Moreover, while a few species are provided with empty membranous appendages, none show any indication of the evanescent helicoid appendages ascribed by Léger & Gauthier (1935) and by Gauthier (1936) to different members of the Harpellaceae, a family of conidial phycomycetes occurring on the peritrophic membrane and rectal cuticle in aquatic larvae of various insects. The predaceous fungi under consideration, together with some obviously related parasitic species, fourteen in number, have accordingly been assembled in a family erected especially for them, the Zoopagaceae.

Stylopage, one of the three predaceous genera in the family, is distinguished by the production of conidia on erect conidiophores. It includes two nematode-capturing species in *S. leiohypha* and *S. hadra*. The former has so far been recorded only from Florida soil, while the latter, after its original description from leaf mould collected near Washington, D.C., has been reported also from Hawaii by Linford (1937), from France by Comandon and de Fonbrune (1938), and from England by Duddington (1940). Six members of the genus, *S. araea*, *S. cephalote*, *S. haploe*, *S. lepte*, *S. rhabdospora*, and *S. rhynchospora*, subsist on terricolous amoebae. *S. scoliospora* likewise lives on amoebae, but occurs under conditions more nearly aquatic than terrestrial.

In the genus *Acaulopage* are collected eight amoeba-capturing members of the Zoopagaceae that bear aerial conidia on very short sterigmata arising from prostrate hyphae. *A. rhaphidospora* is a very delicate fungus with needle-shaped conidia. *A. ceratospora*, *A. cercospora*, *A. macrospora*, *A. marantica* and *A. rhicnospora* show convergence with the predaceous series of hyphomycetes in giving rise to conidia provided with a single empty appendage. Several empty appendages are regularly borne on the conidia of *A. acanthospora* and *A. tetraceros*.

The genus *Zoopage* is distinguished by the development of conidia in chains—a manner of sporulation not known among the predaceous hyphomycetes. It includes one species, *Z. tryphera*, that subsists on a testaceous rhizopod. Soil-inhabiting amoebae are used as prey by the remaining six species, *Z. atractospora*, *Z. cladosperma*, *Z. mitospora*, *Z. nematospora*, *Z. phanera*, and *Z. thamnospira*.

A phycomycete which captures nematodes through adhesion to unseptate mycelial hyphae after the manner of *Stylopage leiohypha*, but which from its reproduction by means of intercalary chlamydospores rather than by means of aerial conidia cannot well be referred to any genus yet erected in the Zoopagaceae, has been presented by Drechsler (1933*c*, p. 269, Fig. 15 C, D; p. 270, lines 7–19) in synoptic figures and brief text. (See also Addendum, p. 26.)

III. THE PREDACEOUS WATER MOULDS

Apart from the fifty predaceous forms belonging in the two mainly terrestrial series already considered, three aquatic phycomycetes habitually capturing rotifers have been made known in the literature. More than two decades after the publication of Zopf's paper on *Arthrobotrys oligospora*, Sommerstorff (1911), working in

Austria, described a fungus whose long continuous mycelial hyphae, measuring about 6–7μ in width, he found growing along algal filaments in water cultures. These hyphae bore at nearly right angles to their axes and at variable but usually short intervals numerous spur-like branches having an average length of 20μ and an average width of 3μ. The distal portion of each branch was filled with protoplasmic material of such dense texture that it appeared refractive and of almost homogeneous texture. When rotifers referable to the genera *Salpina*, *Metopidia*, *Colurus* and *Monostyla*, made oral contact with the densely filled apices they were held despite their violent struggles to escape. Protozoans were also occasionally captured, and once, too, a specimen of a *Gastrotricha*. Though obviously capture was effected through adhesion, it was found that previous to contact with the mouth of a rotifer the branches were not sticky; wherefore the conclusion was reached that adhesive material must be formed as the result of a stimulus from the oral parts. Following capture the branch was found to extend into the prey a stout prolongation, which, after penetrating some distance, ramified to form a system of assimilative hyphae spreading widely through the fleshy body. On appropriation of the animal's substance the contents of the assimilative system were withdrawn backward through the infective branch to permit further growth of the axial filament. Larger rotifers became filled with coarser assimilative hyphae that, instead of yielding their contents to the parent filament, would send eruptive branches through the integument to ramify externally. A clump of amoeboid cells resembling zoospores were once observed at the open tip of an empty eruptive branch. These cells soon rounded up and secreted a wall. In the belief that the fungus probably produced zoospores, it was recognized as a phycomycete, and under the name *Zoophagus insidians* presented as a new species in a new genus. Because it lived like an alga in pure water Sommerstorff held that his fungus could not well be classed as a saprophyte. Nevertheless, he considered the occurrence of long pieces of mycelium devoid of animals to demonstrate some capacity for saprophytic absorption of nutrients from the surrounding water, even while recognizing that this capacity was certainly insufficient to sustain life. Later Sommerstorff (1912) expressed the view that the fungus could not be classed as a parasite, just as the Venus fly-trap could not appropriately be classed as a parasite on insects, or man as a parasite on cattle. The feeding relationship of carnivorous phanerogamic plants and of the two fungi *Arthrobotrys oligospora* and *Zoophagus insidians* he held comparable to that of animals, and accordingly designated it by the term 'bestialism'.

Mirande (1920) reported the occurrence of *Zoophagus insidians* in France. His findings concerning the vegetative morphology and predaceous behaviour of the fungus appear in general agreement with those of Sommerstorff. Like the Austrian investigator he recognized the means of capture in adhesive material at the tip of the short digitate branch, and further agreed that this material played no part until after the animal itself had supplied some necessary stimulus. More specifically, his observations indicated that a reducing substance in the thick cap of the branch became instantly mucilaginous and adhesive when it was acted upon by a juice—possibly a digestive juice—from the animal. With regard to the evidence on which the fungus had been assigned to the Saprolegniales, Mirande stated that in his material also, cysts about 10μ in diameter had been seen, but that whenever conditions were favourable for observation, the bodies were always determined to belong to a chytridiaceous form. Further, he did not share the view that the fungus retained some little capacity for saprophytic existence, but regarded it as an obligate parasite. In his hands it had consistently failed to grow after being transferred to various substrata, thereby revealing an exclusiveness in choice of nourishment little comporting with facultative parasitism. He had never seen a filament devoid of captured animals that was not found to be connected with some hypha which had taken prey. In his cultures abundance of filaments was always correlated with abundance of rotifers.

Sommerstorff's observations on the mycelial morphology and predaceous behaviour of *Z. insidians* were confirmed also by Arnaudow (1921) in a paper reporting studies on Bulgarian material of the fungus. In this paper two types of asexual reproduction, one yielding zoospores, the other yielding gemmae, were described. Zoospore formation was set forth as being accomplished by discharge of protoplasm from portions of mycelium, and conversion of the discharged mass into biciliate zoospores ranging in number from one to more than twenty. After a period of motility these zoospores came to rest, rounded up, and encysted; the cysts subsequently permitting their contents to escape as motile zoospores similar in structure to those from which they were derived. The gemmae were characterized as well-differentiated bodies, measuring 200–240μ in length by 10–14μ in width, that are produced at the tips of mycelial filaments from which they later become disarticulated.

Gicklhorn (1922) studied *Z. insidians* both at Graz in Austria, where Sommerstorff had discovered the fungus, and at Zagreb in Croatia. He observed only rotifers of the group Loricatae, including members of the genera *Colurus*, *Distyla*, *Metopidia*, *Monostyla*, *Salpina*, and *Squamella*, to be captured by the fungus. When animals with more robust ciliary apparatus, such as are found in the genera *Brachionus*, *Noteus*, *Anurea*, *Rotifer* and *Philodina*, were put into cultures of the fungus, they consistently remained unharmed. Treatment of the mycelium with various reagents and stains led Gicklhorn to the conclusion that a mucus capable of extraordinarily rapid swelling was present as a recognizable plug within every branch capable of capturing prey, and that after a stimulus from an animal had taken effect the mucus made up an external cap, sometimes of yellowish coloration, surmounting or enveloping the distal portion of the branch. However, he did not believe adhesion to be operative during the initial stage of capture. According to his observations a rotifer was captured only when in roving about it encountered a branch head on, so that the branch came to be directed straight into its gullet. This manner of contact resulted in a sudden retraction of the ciliary apparatus, whereby the branch was intruded into the animal and firmly engaged by the retracted parts. Thereupon in 10–30 min. the branch exuded all its adhesive material, effectively gumming and disorganizing the ciliary apparatus, which constitutes the animal's chief means of locomotion.

Gicklhorn never observed captured rotifers to be invaded while still alive, but always found extension of haustorial filaments delayed until the animals had died. Such behaviour he considered antithetical to that of true parasites. Evidence of facultative saprophytism came to light also in his successful cultivation of *Zoophagus insidians* in media devoid of rotifers. He obtained abundant development of the fungus in Raciborski's solution, and was able to keep it alive more than two months in boiled filtered water originating from sources in which it was known to occur naturally. In view of the striking increase in growth resulting from capture of animals, and in view, besides, of the structural adaptations for capture, he held the fungus in a broad sense comparable ecologically to the insectivorous phanerogams, but in a stricter sense comparable only with the one other fungus then known to capture animals, namely, *Arthrobotrys oligospora.*

Gicklhorn distinguished four types of development concerned in the asexual reproduction and vegetative propagation of *Zoophagus insidians*: (1) Formation of conidia, (2) production of swarm sporangia, (3) abstriction of stout hyphae, and (4) formation of gemmae. The first-mentioned type of reproduction was described as coming about through eruption from a captured rotifer of a short hypha, 5–10μ wide, that abstricted at its tip spherical conidia, about 15μ in diameter, which collected in a capitate or botryoid cluster. The swarm sporangia were set forth as being of spherical shape, and as being formed always within captured animals. They gave rise endogenously to numerous biciliate zoospores imbedded in a gelatinous material; both the spores and their matrix being discharged at maturity into the surrounding water by way of a protruding distal papilla. The zoospores thus discharged later germinated by the production usually of a single delicate germ tube that soon gave rise to predaceous branches. The type of purely vegetative multiplication enumerated third was described as being accomplished when conspicuously wide hyphae grew out through the integument of a captured rotifer, and were then abstricted, or, perhaps more accurately, delimited by insertion of a basal septum; the delimited part germinating later by production of an axial filament. The bodies interpreted as gemmae consisted of globose expansions intercalated in axial filaments in positions near captured rotifers. Furthermore, some material from Zagreb gave indications of a sexual stage productive of solitary oospores. Gicklhorn believed that the morphological features set forth by him justified assignment of the fungus to the Pythiaceae.

On studying material of *Z. insidians* from the vicinity of Munich, Germany, Arnaudow (1925) supplemented his earlier observations on Bulgarian material. He explained now that the clavate cylindrical bodies, measuring as much as 260–400μ in length and as much as 14μ in width, which he had designated as gemmae, corresponded to neither of the two types of structures known under that term among the Saprolegniaceae—that is, they did not represent arrestment stages in the development of oogonia or sporangia, nor did they consist of portions of vegetative hyphae delimited by septa. The bodies in question were always formed singly at the tip of an axial filament, from which they became disarticulated through median fracture of their very slender attachment. After development of one gemma the supporting hypha would often resume growth by putting forth a short subapical prolongation on which a second gemma would then be formed; repetition of the process resulting in a rachiform termination from which as many as ten gemmae had been abjointed successively, each abjunction being recorded in the presence of a tapering sterigmatic tip. The gemmae germinated usually by producing a mycelial filament which would give rise to predaceous branches, though sometimes predaceous outgrowths would be put forth directly from the reproductive bodies. Even when transferred to pure water the gemmae were never observed to serve as zoosporangia.

In a few mounts prepared from Bavarian material of *Z. insidians*, Arnaudow observed sexual reproduction. According to his description of the process, oogonia and antheridia arise terminally on lateral branches from axial hyphae. These hyphae individually were either wholly male or wholly female in character. Sexual apparatus was found mostly where filaments of opposite character crossed one another. Owing to optical difficulties it could not be determined whether the diclinous relationship was expressive of a dioecious condition. Formation of oögonia and antheridia began simultaneously, though only after the branches had come in contact with each other. Development of the female organ, at least, was initiated by deposition of a delimiting wall. Fertilization was not followed in detail. The mature oogonium was stated to have a brownish membrane, and to contain a single oospore.

Arnaudow held that the sexual reproduction of the fungus argued in favour of membership in the Pythiaceae rather than in the Saprolegniaceae. He considered membership in the Pythiaceae to be sustained also in the type of zoospore development described by him earlier from Bulgarian material, and later again observed in Bavarian material. As far as he was able to ascertain, repetitional swarming of zoospores originally fashioned from protoplasm within a vesicle was not known in other fungi; wherefore he reasoned that the zoosporangial stage observed by him could not well belong to some alien aquatic fungus, though at the same time he admitted that its connexion with *Z. insidians* had not been definitely established. He interpreted Gicklhorn's conidia as probably representing encysted zoospores, and considered Gicklhorn's gemmae to be malformations of infrequent occurrence and meagre significance. As his attempts to cultivate *Z. insidians* in the absence of rotifers did not reveal any capacity for substantial saprophytic growth, Arnaudow held the fungus essentially dependent for its subsistence on capture of prey.

The presence of *Z. insidians* in the United States was adequately recorded for the first time by Sparrow (1929), who found the fungus in some algal material originating from Maine. Since this author's discussion of reproduction, sexual and asexual, appears to be based on Arnaudow's papers, it appears probable that he may have dealt mainly, if not exclusively, with the vegetative stage. Because of the type of zoospore development attributed to the fungus, Sparrow regarded it as being closely related to the species of *Pythium* with filamentous sporangia. The production of gemmae by *Zoophagus insidians* he considered not necessarily incompatible with this view, inasmuch as species of *Pythium* with filamentous sporangia, if investigated more closely, might perhaps likewise be found giving rise to such bodies.

Valkanov (1931 *a*) found *Zoophagus insidians* twice near Sòfia, Bulgaria, and was able to cultivate it in the laboratory for months. He remarked that his observations on the fungus enabled him to confirm many of Arnaudow's statements, though without specifying to which phases of development the confirmation applied. Thus it is not evident that he corroborated Arnaudow's findings relative to the production of zoospores, though he (Valkanov, 1931 *b*, 1931 *c*, 1932) accepted these findings in bringing forward a rather unorthodox taxonomic interpretation of them. To Valkanov the mode of zoospore formation described by Arnaudow did not imply even moderately intimate relationship to *Pythium*, for he believed it failed to embody the feature generally held most distinctive of this genus—complete suppression of a primary zoospore generation homologous to the perfectly developed primary zoospores of *Saprolegnia*, and to the individualized but imperfectly ciliate or wholly unciliated zoospores of *Achyla*. If manner of sporangial evacuation was to be considered of decisive taxonomic significance, he believed *Zoophagus* to merit recognition as a peculiar type, referable in Scherffel's series to a place preceding the type represented by *Saprolegnia*. He was confident that *Zoophagus insidians* belonged in the Saprolegniaceae, together with three other rotifer-destroying phycomycetes, *Sommerstorffia spinosa*, *Synchaetophagus balticus* Apstein (1910) and *Hydatinophagus apsteinii* Valkanov (1931 *c*). In his opinion their lack of gemmae did not militate against assimilation of these phycomycetes to *Zoophagus insidians*. As Arnaudow's very brief remarks on the later development of sexual apparatus in *Z. insidians* include no mention of periplasm, Valkanow assumed the fungus developed no periplasm, and offered the assumption as further proof of an intimate relationship in the Saprolegniaceae rather than in the Pythiaceae.

Recently Arnaudow (1936) reported that experiments and observations on the development of *Z. insidians* failed to reveal chemotropism, geotropism, or heliotropism in the axial hyphae of the fungus. Growth and direction of these hyphae were found governed by autotropism in the sense of Pfeffer. If axial hyphae, which are arranged at right angles to one another, have their growth disturbed by terminal injury, they give rise to one or more, rarely several, subterminal hyphae that continue elongation approximately in their original direction.

On studying water cultures of *Nitella flexilis* started from material collected in New Jersey, Karling (1936) observed *Zoophagus insidians* and an obviously related rotifer-capturing form of more delicate mycelial habit, which he described as a congeneric species under the binomial *Z. tentaclum*. The extended branching hyphae of this form measure 3–6μ in width, and give rise at nearly right angles to numerous lateral branches 15–35μ long and 3–5μ wide. These branches bear at the apex one to five, usually four, divergent tentacles—distinctive processes often somewhat swollen at the base, regularly terminated by a small knob, and measuring 10–17μ in length by 1·5–2μ in width. When a rotifer engulfs the distal portion of a tentacle it is securely held and soon invaded by a coiled or tangled haustorial system, arising either from the apex of the lateral branch or from the knob at the tip of the tentacle. The manner in which capture is affected remains uncertain. In no instance was a tentacle seen to exude a droplet of adhesive fluid. Nor did Karling see any drop of adhesive exudate on the lateral branches of *Z. insidians*, such as had been reported, according to his account, by Sommerstorff, Arnaudow, Mirande, and Sparrow.

As to asexual reproduction in *Z. insidians*, Karling observed the fungus to produce numerous elongated spindle-shaped gemmae or conidia, measuring 40–80μ in length and 3–6·5μ in width. The individual conidium in this species, as in *Z. insidians*, arises singly at the tip of a long mycelial hypha. During its period of growth it remains connected to the hypha by a narrow isthmus. Disarticulation is accomplished through withdrawal of contents from a short median portion of the isthmus, deposition of retaining walls by the separated protoplasts, and fracture of the evacuated membranous connexion. Following repeated subapical elongation of the hypha, additional conidia may be produced in acropetal succession; so that in its terminal portion an old conidiophore may bear a number of acutely pointed peg-like branches. Propagative bodies outwardly somewhat similar to the conidia are often formed in old cultures of *Z. tentaclum* when protoplasmic masses occupying isolated portions of hypha deposit retaining walls at their ends. Karling found no zoosporangia, zoospores, oogonia or antheridia in *Z. tentaclum*, and believed that this fungus will doubtless be found to have the same type of sexual and asexual reproduction as *Z. insidians*.

Taxonomic opinion on the genus *Zoophagus* has undoubtedly been influenced by the general parallelism between the type species, *Z. insidians*, and an aquatic rotifer-capturing fungus that clearly belongs in the Oomycetes. The fungus in question was discovered in association with *Z. insidians* near Sofia by Arnaudow (1923), who described it under the name *Sommerstorffia spinosa*. With respect to manner of attack, it appears to be predaceous only in part, as invasion of living rotifers sometimes takes place following infection by the germination of spores that have entered by way of the oral opening. After an unseptate assimilative hypha has been developed within the fleshy host, a small external mycelium devoid of septa grows out usually from the anterior portion of the integument. On this mycelium are borne one to six straight or somewhat curved, rather acutely pointed, stiff branches, 0·1–0·15 mm. long and up to 8μ wide, which, when orally engulfed by rotifers, operate as predaceous organs. From the posterior region of an invaded animal the internal assimilative system often puts forth a hypha by way of which it discharges the uniseriate protoplasmic lumps that in the meantime have been fashioned from its contents. The discharged protoplasts encyst at the mouth of the evacuation tube; the cysts, 10μ in diameter; later emitting biciliate swarm spores that come to rest after a period of motility and produce, individually, a very short germ tube or infection tube. Oogonia, provided with large blunt protuberances, develop terminally on external hyphae; at maturity these organs contain solitary spherical oospores up to 22μ in diameter. Because of its manner of zoospore formation Arnaudow referred the fungus to the Saprolegniaceae, close to the genus *Aphanomyces*. With this disposition Sparrow, who was able to confirm the *Achyla*-like manner of zoospore production in some material of *Sommerstorffia spinosa* originating from Massachusetts, concurred. More recently the fungus has been reported from the United States also by Karling, who found it in the same material that yielded *Zoophagus insidians* and *Z. tentaclum.*

IV. BIOLOGICAL CONTROL OF PATHOGENIC NEMATODES BY PREDACEOUS FUNGI

In view of the destructive efficacy of many nematode-capturing fungi, it is not surprising that these organisms have been investigated with regard to their possible utilization for the control of nematodes harmful to the health or economic well-being of man. Zopf, as has been noted, used the wheat-cockle eelworm in testing out the predaceous capabilities of *Arthrobotrys oligospora.* While evidently he was impelled to this choice of experimental prey mainly by the circumstance that the wheat parasite, unlike coprophilous eelworms, could be obtained free of troublesome contaminating micro-organisms, he nevertheless manifested a recognition of the utilitarian interest attaching to his studies by suggesting that *Heterodera schachtii* Schmidt, too, might have an enemy in *Arthrobotrys oligospora.* Drechsler (1937*b*) rather incidentally cited *Aphelenchus avenae* Bastian and *Cephalobus elongatus* de Man as examples of weakly parasitic nematodes that were destroyed by predaceous hyphomycetes in his agar plate cultures. Goodey (1938), working in England, observed the stem eelworm, *Anguillulina dipsaci* (Kühn) Gerv. & v. Ben., being destroyed in diseased leaves of *Calceolaria integrifolia* L. as well as in a decayed crown of *Saxifraga cotyledon* L. by a retiary fungus that he identified as *Arthrobotrys oligospora.*

Linford (1937), working in Hawaii, investigated the possibility of reducing root-knot injury in pineapple fields by encouraging development of organisms destructive to nematodes. A series of laboratory and glasshouse tests showed that when chopped pineapple plant material was mixed with naturally infested field soil the ensuing decomposition resulted in an accelerated multiplication of free-living eelworms, which in turn led to such increased development of natural enemies that the numbers of motile nematodes, including the larvae of the root-knot parasite, *Heterodera marioni* (Cornu) Goodey, were greatly reduced. Admixture of pineapple plant material to soil in quantities equivalent to application of 50, 100, 150, or 200 tons per acre resulted in marked and statistically significant reduction of galls produced on susceptible test plants. Examination of Hawaiian field, garden, and forest soils revealed the presence of more than twenty nematode-destroying fungi, among which were included forms similar to, or identical with, the predaceous species *Arthrobotrys oligospora*, *A. musiformis*, *A. dactyloides*, *Dactylella ellipsospora*, *D. gephyropaga*, *Dactylaria thaumasia*, and *Stylopage hadra.* According to a later summary Linford & Oliveira (1938) recognized eleven nematode-trapping fungi among fifty-two natural enemies of the root-knot parasite.

Linford *et al.* (1938) gave results of further experiments showing marked reduction in root-knot infestation from addition of pineapple plant material to soil taken from pineapple fields. Comparable beneficial results were obtained by addition of sugar and of a coarse grass, *Panicum barbinode* Trin. When pineapple plant material was dried in an oven and wetted before being incorporated in the soil, its efficacy for reducing root-knot infestation was augmented. It was suggested that various field treatments for reducing nematode infestation, such as deep mulching with plant refuse, application of bulky organic fertilizers, and green manuring, might owe their beneficial effects to increased development of predaceous and parasitic soil micro-organisms.

Linford & Yap (1938, 1939) studied the capabilities of five predaceous hyphomycetes, *Arthrobotrys oligospora*, *A. musiformis*, *Dactylella ellipsospora*, *Dactylaria candida*, and *D. thaumasia*, for protecting potted pineapple plants against damage from infection by larvae of the root-knot nematode. In their tests relatively uniform quantities of newly hatched root-knot larvae and of fungus culture were added to abundantly replicated and appropriately randomized pots of sterile soil planted with vegetative pineapple slips originating from a single clone. A series of pots free of nematodes and a series of pots infested with the root-knot parasite provided appropriate controls. After the slips had grown 15 months the predaceous fungi present in the roots were identified, and various determinations were made relative to the amount and character of normal plant growth as well as to the quantity and distribution of gall development. Despite a heavy chance infestation of predaceous fungi the tests showed that *Dactylella ellipsospora* restricted nematode injury to a moderate but statistically significant degree. The feebler performance of the other four predaceous hyphomycetes, some of which are more aggressively predaceous than *D. ellipsospora* in agar cultures, was believed indicative of lesser adaptation to the particular conditions prevailing in the experimental pots. Accordingly the conclusion was reached that a proper evaluation of predaceous fungi as agents for biological control of plant-parasitic nematodes must be based on soil tests carried out with suitable host plants.

The feasibility of employing predaceous fungi in biological control of various parasitic nematodes of concern in human and veterinary medicine has recently been given much attention by several French investigators. As larvae of such pathogenic nematodes are sensitive to desiccation and have relatively little capacity for penetrating agar media, Roubaud & Deschiens (1939*a*, 1939*b*) employed a procedure whereby the animals were placed into moist recesses made by removing slabs of substratum from actively growing agar-plate cultures of predaceous fungi. Rhabditiform larvae, stercoral adults, and strongyloid larvae of *Strongyloides füllborni* Linstow, an intestinal parasite of the chimpanzee, *Anthropopithecus troglodites*, induced development of predaceous organs and were being captured within 24–48 hr. after they had been deposited in Petri-dish preparations of *Arthrobotrys oligospora* and *Dactylella bembicodes*. Likewise, development of predaceous organs, mainly again on mycelium spanning the recesses, and capture of prey ensued in 36–48 hr. after rhabditiform and strongyloid larvae of *Ancylostoma duodenale*, a well-known intestinal parasite of man, had been deposited in preparations of the same two hyphomycetes. Development of predaceous organs and capture of prey followed also when to Petri-dish preparations of the two fungi were added either infectious strongyloid larvae of *A. duodenale*, or strongyloid larvae of *Strongyloides* sp., an intestinal nematode of the baboon.

When Roubaud & Descazeaux (1939) added larvae of strongyloid nematodes parasitic on the horse, belonging to the genera *Strongylus* and *Tribonema*, to agar cultures of *Arthrobotrys oligospora* and *Dactylella bembicodes*, the very active strongyloid specimens were captured and consumed in quantity. *D. ellipsospora* showed similar though lesser predaceous capabilities in relation to these parasites. However, the very sluggish rhabditiform larvae apparently evoked very little development of predaceous apparatus.

Descazeaux (1939*a*), in determining that strongyloid larvae of the genera *Strongylus* and *Tribonema* were captured by *Arthrobotrys oligospora* and *Dactylella bembicodes*, made use of nematodes newly hatched from a pasty mixture of dung and powdered charcoal. The mixture was kept confined in crystallizing dishes covered with inverted bases of Petri-dish cultures of the two fungi. Several strips of moist paper placed vertically in the containers provided passageways suitable for migration of newly hatched animals from the dung paste to the agar cultures. Predaceous organs, the formation of which began 24–36 hr. after the relatively early arrival of rhabditiform larvae, operated especially effectively against the tardier strongyloid larvae, with the result that in 20 days all the larvae that emerged from the dung were exterminated. In a later paper Descazeaux (1939*b*) reported that larvae of the family Trichostrongylidae, which are parasitic in the digestive tracts of cattle and sheep, were captured and killed in predaceous apparatus formed after their addition to cultures of *Arthrobotrys oligospora* and *Dactylella bembicodes*. Likewise, when conidia of either fungus were added to water containing such larvae, predaceous organs were formed by means of which the animals were captured and killed.

According to Deschiens (1939*a*) when larvae of *Oesophagostomum bifurcum* Creplin, obtained through sedimentation and centrifuging of excrement from a baboon, *Papio sphinx* Geof., and strongyloid larvae, obtained through similar treatment of excrement from a cow, were placed in moist recesses made by excising slabs from agar plate cultures of *Arthrobotrys oligospora* and *Dactylella bembicodes*, organs of capture were formed whereby the animals were destroyed. Subsequently, Deschiens (1939*c*) reported that the two fungi were operative also in capturing and consuming larvae of *Dictyocaulus filaria*, the causal agent of verminous bronchitis in sheep and goats. While both hyphomycetes destroyed the active animals derived from the second and third moults of the bronchial parasite, *Dactylella bembicodes* showed a more effective predaceous attack in destroying also the feebly motile larvae derived from the first moult.

Attention has been given to the cultivation of predaceous fungi in sufficient quantity for extensive application to areas infested with pathogenic nematodes. Deschiens (1939*a*, 1939*b*, 1939*d*, 1940) reports development of *Arthrobotrys oligospora*, *Dactylella ellipsospora*, and *D. bembicodes* in large quantity on moistened grass stems that had been sterilized by exposure to steam at a temperature of 140° C. for 30 min. On this graminaceous substratum *Arthrobotrys oligospora* and *Dactylella ellipsospora* produced an abundance of conidia in 10 days, though *D. bembicodes* required a month for sporulation. The sporulating material, on being run through a grinder, was converted into a fine powder suitable for extensive application by dusting. Liquid medium containing 90 parts of distilled water to 10 parts of meat broth, straw decoction, or hay decoction, and sterilized for 30 min. at 140° C., yielded copious mycelium 5 days after inoculation with *Arthrobotrys oligospora* and *Dactylella ellipsospora*. The culture thus obtained, after being shaken up with glass beads, became an aqueous suspension of mycelial fragments ready for extensive application by means of spraying machinery.

When square pieces of turf were taken indoors and regularly sprayed with this aqueous suspension, the fungi formed a mycelial weft at the surface of the soil as well as on vegetable debris elsewhere, and sporulated. Some hyphae penetrated into the soil, others ensheathed the basal portions of grass stems and clover stems. Comparable though less copious development took place in sod or turf exposed to the weather outdoors. In response to drought the fungi disappeared at the surface but penetrated into the moister portions of soil. On partial flooding considerable growth was obtained in the water, and about the bases of the culms. Capability for extensive outdoor growth was made evident also in development of the predaceous hyphomycetes on the surface of fecal mire and dung (Roubaud & Deschiens, 1939*b*; Descazeaux, 1939*a*). A further observation by Descazeaux that the hyphomycetes are able to develop in water containing organic materials was held to imply that pools, puddles, and flooded fields—all of which are often infested with nematode larvae—may advantageously be sown with predaceous fungi.

To determine whether applications of predaceous fungi have an objectionable effect on pasturage Deschiens (1939*b*) spread *Arthrobotrys oligospora* and *Dactylella bembicodes* on a plot of meadow land. After 50 days this plot, which bore numerous mycelial wefts at the surface of the ground, was fully equal in merit to the control plot that had received no addition of fungus material.

Deschiens and his colleagues admit that possibly even under ordinary natural conditions the predaceous hyphomycetes in some measure carry on destruction of infectious nematode larvae. By providing for increased mycelial development through application of fungus material—the increased development reported by them seems greatly in excess of expectations—they aim to augment the activity of the predaceous hyphomycetes to the point where it becomes effective as an important subsidiary, if not principal, prophylactic factor. Manifestly their treatment by extensive application of fungus material departs markedly in principle from the treatment investigated in Hawaii, whereby large quantities of organic matter are incorporated in the soil. The former treatment would seem to be based on the idea that nematode-capturing fungi are meagrely distributed in nature, and yet are addicted to very aggressive saprophytic development when brought into contact with foul natural substrata; whereas the latter treatment postulates rather that the fungi in question are very generally distributed in soil and decaying materials, that they subsist habitually on nematodes, and that accordingly they can best be stimulated in vegetative development and useful predaceous activity by first supplying them with larger quantities of saprophilous living prey.

V. SUMMARY

Of the fifty-three fungi that have been reported as subsisting by the capture of motile animals, twenty-five are hyphomycetes intimately related to one another, twenty-five are phycomycetes of the family Zoopagaceae, and one, an aquatic form, is definitely a member of the Saprolegniaceae. The remaining two, both of which are aquatic phycomycetes, have most often been considered referable to the Pythiaceae, yet the possibility is not to be ignored that they may belong in the Zoopagaceae.

Among the twenty-five predaceous hyphomycetes are included twenty-one species that capture nematodes, three species that capture testaceous rhizopods, and one species that captures amoebae. Nineteen of the nematode-capturing hyphomycetes employ remarkably specialized predaceous organs—adhesive networks, stalked adhesive knobs, adhesive columnar processes, constricting rings, and non-constricting rings. The twenty-five predaceous Zoopagaceae capture animals through adhesion to virtually undifferentiated mycelial hyphae, twenty-one species preying on amoebae, three species preying on nematodes, and one species preying on testaceous rhizopods. The three aquatic phycomycetes capture rotifers by means of short lateral branches, which, according to most observers, become adhesive on being engulfed in the oral opening of the prey.

Invasion of nematodes by assimilative hyphae of predaceous fungi promptly results in conspicuous fatty degeneration of the animal's musculature and organs. Invasion of amoebae and shelled rhizopods causes relatively little immediate degeneration; the organisms apparently remaining alive until their sarcodic materials have in large part been expropriated.

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VII. ADDENDUM

In a recent paper Drechsler (1941[1]) has presented three additional predaceous fungi as new members of the Zoopagaceae. One of these fungi, which is described under the binomial *Acaulopage stenospora*, offers little novelty, since with respect to morphology it appears rather closely comparable with species previously known. More particularly it resembles *A. rhaphidospora* and *A. macrospora*; and like these congeneric forms subsists by capture of small terricolous amoebae through adhesion to delicate mycelial filaments. The two remaining predaceous fungi reproduce asexually by intramatrical spores, which, though somewhat resembling the conidia of the parasitic zoopagaceous genus *Endocochlus* in manner of development, are to be regarded as chlamydospores. Accordingly a new genus, *Cystopaga*, is erected to provide for forms that produce chlamydospores instead of conidia. *C. subtilis* captures specimens of a testaceous rhizopod closely related to or identical with *Lecythium mutabile* Bailey (Hopk.), capture being effected through adhesion to slender mycelial hyphae. *C. lateralis* resembles the unnamed phycomycete previously mentioned (p. 12) in capturing nematodes through adhesion to relatively stout mycelial filaments, but differs from that fungus in producing its chlamydospores always laterally, never in intercalary positions.

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[1] Four phycomycetes destructive to nematodes and rhizopods. *Mycologia*, **33**, 248–69.

ARTIFICIAL PARTHENOGENESIS

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I. INTRODUCTION

SINCE the discoveries by Morgan (1896–1900), Mead (1898), Hertwig (1896) and Loeb (1899, 1900) that development could be initiated in unfertilized eggs by artificial means and that normal larvae could develop from artificially activated eggs, there has appeared an enormous number of publications on the subject of artificial parthenogenesis. The number of investigations increased to a maximum during the first two decades following the initial discoveries, then slumped off considerably during the third decade and is again on the upgrade in recent years. This revival of interest appears to be occasioned not so much by attempts to increase the variety of animals subject to artificial parthenogenesis, or to find new methods of activation, but to apply the newer practical and theoretical findings to questions unanswerable to the earlier investigators and to examine problems only partially treated or overlooked in the older work. The present article is an attempt to review the more recent work. The latest detailed accounts of artificial parthenogenesis are those of Morgan in 1927 and Dalcq in 1928. A recent text by Just (1939) contains an interesting re-examination of the older work but does not present any of the newer material. Since these books, together with Lillie's (1919) and Loeb's (1913) and the summary of methods by Harvey (1910), contain very complete references, there is no need to refer here to any of the earlier experiments except those directly pertinent to the discussion.

The prime interest in artificial parthenogenesis has for some time resided in the supposition that one could thereby unravel the problems of fertilization. But, due to the great numbers and diversity of treatments that have been discovered, the idea that the parthenogenetic agent imitates the sperm or represents the effective component of the spermatozoon had gradually been abandoned. It is generally recognized now that the spermatozoon is one of a number of diverse agents by means of which the egg may be activated. Interest in the activation problem has turned towards attempts to find the common factor involved in the treatment by the diverse agents. While the discovery of artificial parthenogenesis did not at once present a solution of the problems of activation, it tremendously enlarged the scope of the attack by substituting for a complex agent, the spermatozoon, simple chemical and physical agents. Thus, activation of the egg comes to be regarded as part of the general problem of cell stimulation, and it is from this point of view that some of the most interesting recent advances have been made.

The interest in artificial parthenogenesis has not, however, been confined to the problem of activation. In normal fertilization the spermatozoon contributes, besides the activating stimulus, a set of chromosomes which embodies the paternal contribution to the genetic make-up of the zygote, a central body which in some way gives rise to the machinery for cell division and, in some cases, it determines through its point of entrance into the egg the plane of bilateral symmetry of the embryo. In the absence of these contributions of the spermatozoon the development of the artificially activated egg may diverge considerably from the normal. Thus artificial parthenogenesis comes to be a useful tool in the examination of questions concerning the relative roles of nucleus and cytoplasm in development and heredity, the mechanism of cell division, the problem of embryonic determination, sex determination, etc. Also it affords to the geneticist the opportunity of producing genetically homozygous types in less time than ordinarily, and, to the physiological embryologist, opportunities for examining the effect of controlled alterations in the development of the egg on its metabolic changes.

II. THE PRODUCTION OF NORMAL EMBRYOS

1. *Advanced stages*

The extensive investigations of the early workers have shown that in practically all the main groups of animals normal development can be obtained by artificial activation of the eggs. In several cases the embryos have been reared to the adult condition. In sea-urchins and starfish (Delage, 1904, 1908, 1909; Shearer & Lloyd, 1913) a small number of larvae have been reared through metamorphosis. Several dozen metamorphosed frogs are now on record from the work of numerous investigators (Bataillon, 1910; Herlant, 1913; Levy, 1913, 1920; Loeb & Bancroft, 1913; Loeb, 1916, 1918, 1921; Hovasse, 1922; Parmenter, 1932, 1933; Kawamura, 1939*b*), and many of these have been reared to sexual maturity. In the silkworm moth sexually mature adults as well as caterpillars have been readily obtained in large numbers after activation by heat and by acid (Sato, 1931; Koltzoff, 1932; Astaurov, 1936).[1] In fish there are two reports on rearing parthenogenetic individuals to an advanced stage. Shad eggs activated by a weak galvanic current in a salt solution were raised to a practically full-grown condition on an industrial scale (Lestage, 1934). Young carp up to 8½ months old were obtained by activating the eggs with male human saliva (Kasansky, 1935). In mammals the production of at least seven parthenogenetic rabbits has been reported—six obtained by treatment of the eggs with heat or hypertonicity *in vitro* and implantation in a prepared doe (Pincus, 1939 *a* and *b*), and one by cooling exposed fallopian tubes containing eggs ovulated as a result of pituitary treatment (Pincus & Shapiro, 1940).

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[1] The relative ease with which artificial activation may be obtained in this form may be connected with the natural occurrence of parthenogenesis in many races of *Bombyx mori*, and this is generally offered as a reason for not considering Tichomiroff's (1886) work as the first demonstration of artificial parthenogenesis. The view has been proposed (Jucci, 1928; Manunta, 1936) that all races of *Bombyx mori* possess a capacity for natural parthenogenesis, but differ in degree, the tendency being correlated with polyvoltinism and type of metabolism.

The lack of a greater number of cases of normal parthenogenetic adults is due primarily to the fact that those animals, such as marine invertebrates whose eggs are most easily handled and most frequently used in parthenogenesis experiments, are also the ones that are most difficult to rear in the laboratory. It is clear that, where an experiment yields perfectly normal larvae, the problem of obtaining the adults is principally a matter of culture methods. The production of normal embryos can then be regarded as an adequate criterion for the success of a parthenogenetic treatment.

2. *Frequency of normal development*

One might expect that, as a result of over forty years of investigation, methods should now be known that yield as high a percentage of normal development as does ordinary fertilization. However, that is not the case. Whereas fertilized eggs, with proper care, will practically all develop into normal embryos, artificially activated eggs give in general very low percentages.This is often assumed to be due to failure to find the best agent and optimum treatment for stimulating the eggs of the particular species. It is certainly true that eggs of different species vary greatly in the degree to which they will respond to a particular agent, as do also eggs of one species to different agents. However, the production of normal embryos is not merely a matter of finding the optimum method of stimulation. Even where a particular treatment initiates development in all of the eggs in a manner indistinguishable from that induced by the sperm, only a low percentage may produce normal embryos. This can be attributed in most cases to disturbances in the distribution of the chromosomes, to the absence of a proper division mechanism normally supplied by the central body of the spermatozoon, and in some cases to the lack of a well-established plane of bilateral symmetry. The last-mentioned point will be considered briefly in this section and the others in subsequent sections of this review.

3. *Bilateral symmetry*

The early observations of Newport (1853), Roux, (1887, 1903) and Brachet (1904) showed that in the frog's egg the plane of bilateral symmetry is determined by the point of entrance of the sperm. In some species of annelids and molluscs it has also been reported (Just, 1912; Morgan & Tyler, 1930; Tyler, 1931 *a*) that the sperm is instrumental in determining the bilateral plane of the embryo. In these forms the proportion of normal embryos obtained by artificial parthenogenesis has, in general, been much less than in such forms as the sea-urchin where no such determining influence on the part of the sperm-entrance point appears to exist (Hörstadius, 1939). The fact that normal embryos do develop, even though with low frequency, in eggs of the former type raises the question as to how their bilateral symmetry is established.

Brachet (1911) examined this question in experiments on eggs of the frog activated by the puncture method. In the frog's egg the plane of bilateral symmetry bisects the grey crescent and the position of the latter is determined ordinarily by the entrance point of the sperm.[1] In eggs activated by puncture with a fine needle Brachet found the position of the grey crescent to be independent of the point of puncture. He interpreted the result to mean that the egg itself has a sort of labile bilaterality which expresses itself in the absence of the spermatozoon. He pointed out further that the action of the needle is not quite comparable to that of the sperm, since even the finest is relatively gross and its action crude and especially much more rapid than that of the spermatozoon. It reaches the centre of the egg in a fraction of a second and the egg reacts *en masse* almost simultaneously in all its parts. He suggested using a very fine needle and allowing it to take 10–15 min. to reach the centre of the upper hemisphere of the egg. But as yet such an experiment has not been attempted.

The symmetry question was also considered in some experiments of my own (1931 *a*) on eggs of the gephyrean worm, *Urechis*. It was shown (Taylor, 1931; Tyler, 1931 *a*) in this species that the plane of bilateral symmetry bears a definite relation in the great majority of cases to the entrance point of the spermatozoon. Since there was no indication here of a preferred site of entry into the egg, the entrance point could be considered to determine the bilateral plane. By activation with hypotonic solutions or other agents normal embryos (Fig. 1) were obtained, but the percentage was extremely low. Even when eggs were isolated that underwent normal cleavage, only about 10% gave normal top-swimming trochophores. Among the abnormal embryos there were found a large number (up to 25%) that could be classified as radially symmetrical or nearly so (Fig. 2). These embryos survived well beyond the time at which bilateral symmetry appeared in the normal embryos. The development of these radially symmetrical forms was considered to be an expression of the pattern of organization of the unfertilized egg. In the few embryos that developed normally, bilaterality was assumed to arise "by chance". The term "by chance" is admittedly unsatisfactory. It may be taken to mean that there is open to an originally radially symmetrical egg the possibility of assuming a bilateral organization or one of a number of asymmetric patterns and that in the absence of a directing influence (the spermatozoon) it is a matter of chance which occurs. It may also be taken to mean that movements of morphogenetic substances occur upon activation and in the absence of the influence of the sperm the movements are random, assuming only occasionally a normal bilateral distribution. But since nothing is known about the number of possible asymmetric patterns, nor much about the movements of morphogenetic substances, there is little possibility of testing these views.

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[1] The validity of this relation has been questioned recently by Pasteels (1937).

Another somewhat different view offers the possibility of experimental attack. It may be assumed that the unfertilized egg of the type under discussion is normally radially symmetrical in its general organization, and that it would develop according to that pattern in response to a stimulus that is applied simultaneously to all parts of the surface. No practical method of activation could, however, give exact simultaneity of action on all parts of the egg. Even where eggs are rapidly immersed in a solution of the activating agent or exposed to a temperature change, there is bound to be a difference in the time at which different parts of the surface are reached.

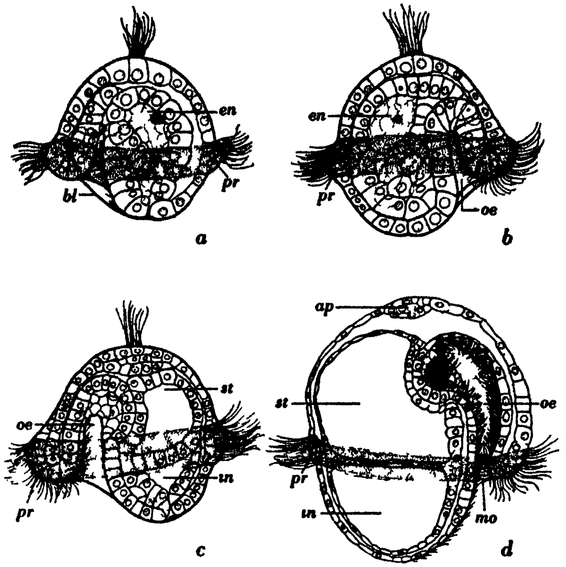

Fig. 1. Normal *Urechis* embryos from eggs activated with hypotonic sea water; *a*, 16-hour trochophore; *b*, 20 hr.; *c*, 24-hr.; *d*, 48-hr. (From Tyler, 1931 *a*.)

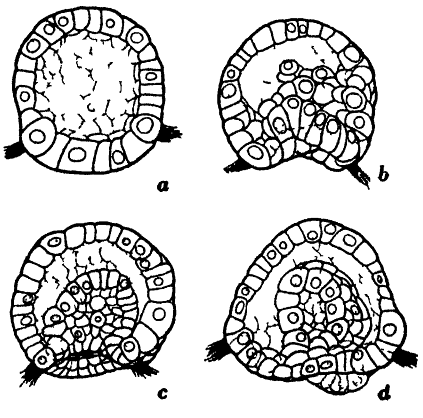

Fig. 2. Radially symmetrical *Urechis* embryos from eggs activated with hypotonic sea water; *a*, 24-hr. "Dauerblastula"; *b*, 22-hr. gastrula; *c* and *d*, 28-hr. (From Tyler, 1931 *a*.)

Depending, then, upon the kind of assymmetry of treatment to which the egg is exposed, departures from the radially symmetric pattern of development would be expected. Where the optimum concentration or intensity of the activating agent grades off from one point on the surface of the egg or where the treatment is applied at one point, bilateral development would be expected. Where the action radiates from two or more (not diametrically opposed) points various types of asymmetry should arise. One would consider then on this basis the puncture method to be ideal for this type of egg. This is not, however, necessarily the case, since divergence from a position normal to the surface and the direction of travel within the egg may very well be factors in the result. Experiments in which the activating agent is applied in the manner of a gradient would be one way in which this view could be tested. Such experiments have not as yet been performed.

III. ORIGIN OF THE CLEAVAGE AMPHIASTER

1. *In fertilized eggs*

In the fertilized eggs of practically all animals the amphiaster for the first division arises by the division of an aster (sperm aster) that forms adjacent to the sperm pronucleus. The numerous observations on the cytology of fertilization have shown few exceptions, such as the case of *Crepidula* (Conklin, 1904), where one pole of the amphiaster is contributed by the egg aster, and the case of the honey bee (Nachtsheim, 1913) and the trematode *Polystomum* (Minouchi, 1936), where both are derived from the egg aster. On the basis of Boveri's (1895, 1900) classical theory of fertilization the sperm aster is derived from the central body (centrosome) of the spermatid and is transmitted to the egg as the midpiece of the spermatozoon. Opposed to this is the view that the sperm aster (and central body) arises *de novo* under the influence of the sperm pronucleus. The evidence bearing on this question is too lengthy to be reviewed here. Mention should be made, however, of the excellent cytological investigations by Pollister (1930), Johnson (1931) and Huettner (1933), who in more recent years have furnished convincing evidence of genetic continuity of the central bodies. Since Boveri's view assumed such genetic continuity, it would appear to be supported by these investigations. But the acceptance of that view does not exclude the possibility that a self-perpetuating cell organ may, under certain conditions, arise *de novo*. In some of the work on artificial parthenogenesis there is evidence of such an occurrence in the case of the central body and aster.

Whereas in normal fertilization, in most animal eggs, no aster arises from the central body which remains after the polar divisions, an egg aster does usually appear as a result of artificial activation. In addition there may arise also accessory asters, the so-called cytasters, first described by Morgan (1899, 1900). These have not been observed in eggs of all animals that have been studied but principally in the echinoderms and the amphibia. In these forms the question as to the origin of the amphiaster has centred about the relative roles of egg aster and cytasters. The problem has been the subject of numerous investigations, and has been discussed in the texts by Wilson (1925), Morgan (1927) and Dalcq (1928). Bataillon, who has done perhaps the most extensive work in this field, has also presented some newer data (Bataillon, 1929; Bataillon & Tchou-Su, 1930, 1934), and has amplified his earlier views.

2. *In artificially activated eggs of some species*

According to Bataillon the amphiaster in the anuran egg activated by the puncture method is derived from a cytaster. He finds that cytasters, capable of forming an amphiaster, arise only when the puncture introduces some foreign nuclear material in the form of leucocytes or other cells or their fragments. A variable number of these asters arise and the principal one (presumably the deepest one) enlarges, receives the egg pronucleus, and divides to form the amphiaster for the first cleavage. The accessory cytasters generally degenerate, but if they are too numerous or too close to the principal aster they may cause disturbances, such as abnormal mitotic figures, etc. That foreign cellular elements are needed is shown in experiments in which the gelatinous coat of the egg, which is normally contaminated with such cells, is removed by means of cyanide treatment. The denuded eggs, upon activation by puncture, form in general no cytasters but only a monaster (egg aster), which undergoes the typical monastral cycles. Occasionally cytasters may later appear and complicate secondarily the monastral cycle. By conjugating with the monaster they can form an "unbalanced pseudo-amphiaster" and participate in an unequal distribution of the chromosomes. It would be of interest to know whether the denuded eggs would form amphiaster-producing cytasters upon introduction of foreign nuclear material. The cyanide evidently has no injurious effect on the egg and is frequently used to remove the jelly upon normal fertilization of frog's eggs. But the possibility is not excluded that introduction of jelly material may be a factor in the result. Bataillon has not reported such experiments, but supports his view with cytological observations showing recognizable lymphocytes, leucocytes, etc., associated with the cytasters.

The origin of the amphiaster in the sea-urchin egg, Bataillon considers to be essentially similar to that in the frog's egg. When activated by a single treatment with a fatty acid or similar agent the egg forms a monaster, as has been shown in a great many of the earlier accounts. While Bataillon does not discuss the relation of the monaster to the central body remaining in the egg after the second polar division, it quite evidently is derived from that body. This egg aster undergoes the usual monastral cycles and the egg cytolyzes in a relatively short time without cell division. When the egg is given a second treatment with hypertonic sea water or a similar agent then cytasters arise, and one of these near the egg nucleus divides to form the amphiaster. Just what happens to the egg aster is not clear from Bataillon's account. The second treatment is applied in general before the monaster appears. One might assume that it is suppressed, or, as a result of the hypertonic treatment, that it develops sooner and acquires the ability to form an amphiaster. The latter seems unlikely on the basis of the results of the type of experiment originally performed by Ziegler (1898) and more recently by Fankhauser (1925–37*b*), Woerdeman (1935), Moore (1937) and Harvey (1934, 1940*b*), in which the uncleaved fertilized egg is separated into two parts, one containing the egg pronucleus, the other the sperm pronucleus. The latter forms a typical amphiaster and divides. The former does not in general divide, but usually forms a monaster, presumably from the egg central body which becomes active in the fragment. Apparently, in normal fertilization, the developing sperm aster suppresses the egg aster. An inhibitory action of this sort has been nicely demonstrated in the constriction experiments of Fankhauser (1925–37 *b*) on the salamander egg. In artificially activated eggs it may be that the cytasters produced by the hypertonic treatment have a similar action on the egg aster.

Bataillon emphasizes in his work the idea of the necessity for a double treatment although from a different point of view from that originally proposed by Loeb. The second factor he considers essential for the formation of an amphiaster-producing aster. This is supplied by the foreign nuclear material in the case of the frog's egg or by the hypertonic treatment in the case of the sea-urchin. The double-treatment notion has been largely abandoned since Just's (1922) demonstration of the efficacy of hypertonic treatment alone. But Bataillon concludes from his own experiments that the treatment involves two factors: namely, alkalinity along with hypertonicity. It seems hardly warranted to accept this conclusion as having very general significance, especially as there are activating agents known, such as heat, ultra-violet radiation, etc., in which it would be difficult to distinguish two agents.

In the egg of the lamprey, Montalenti (1932, 1934, 1936) has succeeded in obtaining normal-looking embryos (gastrulae) by artificial activation and emphasizes the effectiveness of double treatment. He finds the best treatment to be exposure to vapours of chloroform (or alcohol, ether, xylol, etc.) followed by hypertonic solution. The first treatment alone gives an initial response indistinguishable from that obtained upon fertilization, but results in a series of monaster cycles and failure of the "polar plasm" to undergo its normal movements. Cytological observations on eggs subjected to the second treatment are not presented, but the origin of the amphiaster is considered to be similar to that described by Bataillon for the frog or sea-urchin.

In fresh water fish (*Acerina cernua, Perca fluviatilis, Abramis brama, Rutilus rutilus*) Trifonowa (1931, 1934) has made some cytological observations on parthenogenetically activated eggs. A "natural rudimentary parthenogenesis" occurs in these forms when the unfertilized eggs are simply placed in water. They form monocentric mitoses (monasters) and give only abortive cleavages. The blastoderm may spread over the yolk, as in normal development, but this generally occurs without cell or nuclear division. Occasionally nuclear division without cell division takes place, and rarely both occur. By simultaneous action of high temperature (28–30° C.) and distilled water a greater percentage of the eggs undergo parthenogenetic development. Normal cleavage is obtained, but development goes in general only to the stage of blastopore closure. In two cases 13-day-old hatched embryos of dwarf size were obtained. While cytasters are described in the artificially activated eggs, no other information pertinent to the origin of the cleavage amphiaster is given.

Another aspect of Bataillon's views should be mentioned here. He considers the cytasters, or asters in general, to be capable of division only when a nucleus is present. This notion has been held by many investigators. There has been, however, steadily accumulating a good deal of evidence showing not only multiplication of asters in the absence of nuclear material but cell division as well. This work will be considered in the next section.

The question of the origin of the amphiaster has been examined in a number of other marine invertebrates besides the sea-urchin. There the problem is complicated, not by cytasters which have rarely been observed, but by abnormalities in the polar body divisions. It will be discussed, then, in the section on polar bodies and cleavage.

IV. ACTIVATION OF NON-NUCLEATE EGG FRAGMENTS

1. *Earlier work*

One of the most striking phenomenon in artificial parthenogenesis is the formation, in eggs of many species of animals, of cytasters. Soon after their discovery (Morgan, 1896, 1900) the question arose as to their nature, and, more particularly, whether or not they were comparable with the asters of the normal mitotic figures. The early experiments of Wilson (1901), Yatsu (1905) and McClendon (1908) on activation of enucleated egg fragments made it clear that the cytasters were formed *de novo* and not as a result of a very rapid multiplication of the egg-central body. Wilson also showed that the cytasters in the enucleated fragments would increase in number by division which was initiated by the splitting of the central body. But while he often observed cleavage furrows forming about the cytasters, he never obtained complete cytoplasmic division. Since that time there has been a considerable number of investigations concerned with the question of the capacity of cytasters to divide and to cause cell division. These have been reviewed by Dalcq (1931) (see also Dalcq & Simon, 1932), who contributed some ingenious experiments of his own to the problem. More recent work by Fankhauser (1934), Gross (1936), and particularly by Harvey (1936–40*b*), now shows not only that asters are capable of division in the absence of nuclear material, but cell division can also occur. Mrs Harvey's work, which is pertinent to the present discussion, may be briefly described here.

2. *Production and activation of fragments*

By the technique of centrifuging at high speed in isosmotic sucrose-sea water (one part of sea-water containing eggs placed over two parts $0{\cdot}95\,M$ sucrose and partially mixed), Harvey (1936) obtained non-nucleated fragments of unfertilized eggs of the sea-urchin *Arbacia* in great numbers. Since the mixture has approximately the same density as the eggs the latter remain suspended during centrifugation. Under the influence of the centrifugal force the egg contents stratify, the egg elongates, becomes dumb-bell shaped and finally breaks into two parts, as is illustrated in Fig. 3. The light fragments move toward the centripetal end of the tube, the heavy fragments toward the centrifugal end and the unfragmented eggs remain in between. Each type forms a rather sharp layer, which can be pipetted off with practically no contamination. The nucleus is invariably just below the oil layer in the light fragment (*white half*), which is usually slightly larger than the *red half*. Each of the halves can be broken into quarters upon further centrifugation. The *white half* gives a *clear quarter* containing the oil, clear layer and nucleus and a smaller *granular quarter* containing the mitochondrial layer and some yolk. The *red half* gives a *yolk quarter* and a much smaller *pigment quarter*. Since the nucleus remains intact, there is not much likelihood for a portion of it to be contained in any fragment other than the *white half* or the *clear quarter*. Cytological studies (Harvey, 1940*a*) bear out this point. This method of producing non-nucleate fragments is quite evidently superior to, and free from the sources of error (see Boveri, 1918) in, the original method of shaking eggs. It is also considerably easier than the laborious procedure of cutting eggs individually.

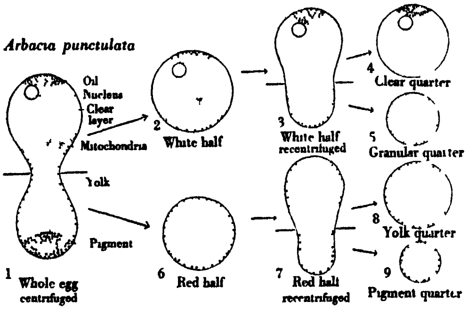

Fig. 3 Stratification and fragmentation of unfertilized eggs of *Arbacia punctulata* by centrifugal force (10,000 g. for about 3 min.). (From Harvey, 1936.)

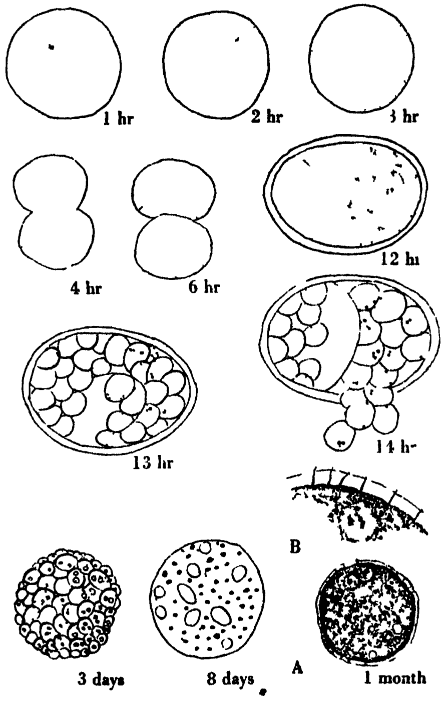

Fig. 4. Cleavage and development of some non-nucleate egg fragments of *Arbacia* activated with hypertonic sea water. (From Harvey, 1936.)

The non-nucleated as well as the nucleated fragments can be activated artificially with the same kind of treatment that is effective for the whole eggs. Mrs Harvey employed principally a single treatment with hypertonic sea water. Of particular interest is the behaviour of the non-nucleated fragments. Upon activation membrane elevation usually occurs, but development may proceed without membrane elevation, as is the case with the intact egg, too. Within an hour or two after activation a single cytaster appears in many of the eggs (Fig. 4; 2 hr.). This is followed by an amphiaster and then division of the fragment between the two asters (Fig. 4; 3, 4 and 6 hr.). Frequently more than one cytaster may arise at the same time in the fragment as they do in the whole egg. The first division may also result in more than two cells. Often, too, the division planes fail to come in or to complete themselves, although the cytasters multiply in the unsegmented fragment (Fig. 4; 12 hr.). This type may sometimes divide into many cells at once just prior to emergence from the membrane. In the best cases the divisions follow one another in a more or less orderly sequence. The cleavages are much slower than in the nucleated fragment, which in turn cleaves more slowly than the fertilized fragment or whole egg.

3. *Extent of development*

The most advanced stage obtained by artificial activation of the non-nucleated fragment consisted of about 500 cells (Fig. 4; 3 days). Some are reported to be normal-looking free-swimming blastulae.

The experiments with *Arbacia punctulata* were repeated (Harvey, 1938) with four other species of sea-urchins, *Arbacia pustulosa*, *Sphaerechinus granularis*, *Paracentrotus lividus* and *Parechinus microtuberculatus*, to examine the possibility that some of these might give further development. These experiments confirmed the earlier ones but did not result in more advanced stages or more normal development. Experiments with the polychaete annelid *Chaetopterus* (Harvey, 1939) gave much poorer results, the artificially activated non-nucleated fragments going no further than the first cleavage. This may be due to lack of the proper treatment for producing cytasters which have not as yet been described in annelid eggs.

The developmental capacity exhibited by the non-nucleated fragment has led Mrs Harvey to raise again the question as to whether or not the nucleus controls only the more specific and differential characters of the organism. But, since a previous effect of the nucleus on the egg cytoplasm such as is exhibited, for example, in the inheritance of coiling in snails (Sturtevant, 1923) cannot be ruled out in the present experiments, no definite conclusions are drawn in regard to this question. The contributions of her experiments are of more importance in connexion with the cytasters.

4. *The* de novo *formation of a self-perpetuating body*

It is clear from the experiments that the cytasters are capable of mutltiplying in the absence of nuclear material. In normal mitoses asters arise in connexion with, and are probably due to the activity of, a self-perpetuating central body. Harvey (1940*a*) does not describe definite central bodies in the cytasters, but Wilson (1901) figured them quite clearly in his cytological observations on activated non-nucleated fragments. Since, as Fry (1933, 1937) has shown, the appearance of the central body may vary considerably, depending upon the cytological technique employed, it is reasonable to assume that the appropriate method would demonstrate distinct central bodies in Harvey's material too. It appears then that the production of a cytaster involves in effect the *de novo* formation of a self-multiplicative, genetically continuous body.

A highly interesting new development bearing on the nature of the central body is the demonstration by Pollister (1939; Pollister & Pollister, 1940) that it may be derived from the part of the chromosome known as the kinetochore, centromere or spindle fibre attachment. He showed in the atypical spermatogenesis of several species of snails of the genus *Vivipara* a close correlation between the increase in number of central bodies and in number of unattached chromosomes. Central bodies (centrioles) appear then to be of the same nature as chromosomal material, and this accords with the similarity in such properties as their reaction with dyes and their appearance in the living condition (see Schrader, 1936). Since, as the experiments on artificial activation of enucleate fragments seem to show, central bodies may be formed *de novo*, it does not appear too fantastic to expect that under the proper conditions chromosomal material or genes may be caused to arise *de novo*. Harvey (1940*a*) has added various substances, particularly those associated with the nucleus, to the activated non-nucleated fragments with the intention of obtaining better development. This did not succeed, nor did she note the formation of nuclei. However, since the egg-cytoplasm itself contains an abundance of such material (see Brachet, 1937; Painter, 1940; Caspersson & Schultz, 1939, 1940), it may not be the addition of more of these substances that is required but rather a method of treatment that would induce the formation of an appropriate site of synthesis in the non-nucleate fragment. Perhaps mutation-inducing agents would be worth examining in this connexion.

5. *Spindle and amphiaster*

In the multiplication of the cytasters and cleavage of the non-nucleated fragment Harvey (1940*a*) observes no spindle formation. This accords with the generally accepted view that the chromosomes are involved in the formation of the spindle fibres, as is well illustrated, for example, in Packard's (1918) observations showing the absence of spindle fibres to X-rayed chromosomes in *Chaetopterus*.

In regard to the origin of the amphiaster in artificially activated whole eggs, the work on the non-nucleated fragments supports the view that it arises from one of the cytasters. On the other hand, the possibility is not excluded that the egg centre may, under certain conditions, regain its capacity to divide. In experiments to be described in the next section on other species of marine animals than the sea-urchin the egg centre does in fact exhibit a renewed capacity for division.

V. POLAR BODIES AND CLEAVAGE

1. *Polar body suppression*

In practically all groups of animals other than the sea-urchin the eggs are shed and ripe for fertilization before they have extruded both polar bodies. In most vertebrates only the first polar body is extruded and meiosis stops at metaphase of the second division. In most invertebrates the maturation divisions stop before the first polar body is extruded, some species going as far as the metaphase of the first division, others remaining in the germinal vesicle (large nucleus of the primary oocyte) stage. Upon fertilization the maturation divisions are, of course, completed normally. But upon artificial activation, particularly with the invertebrate eggs, one or both polar bodies may often fail to be extruded. This has been noted by a great many of the early investigators (see Wilson, 1925, p. 476). The conditions under which it occurs and the subsequent behaviour of the egg have been examined in some recent investigations which will be discussed in this section.

2. *Hypotonic activation in* Urechis

In the echiuroid worm *Urechis caupo* it was found (Tyler, 1931 *a*, *b* and *c*) that polar body suppression occurred upon sub- or supra-optimum exposure to the diluted sea water which was principally employed as the activating agent. The percentage of eggs that are activated increases with time of exposure, as is the case with other agents. With prolonged exposure to solutions not too strongly hypotonic the percentage falls off again to zero. After optimum treatment, that is, exposures resulting in 100% activation, all of the eggs extrude both polar bodies. With the shorter or longer exposures that give lower percentages of activation increasing numbers of eggs are found that fail to extrude polar bodies; while in the hypotonic solutions the eggs simply swell, due to intake of water, and show no sign of activation. In solutions that are not more hypotonic than 45% sea water increase in exposure time results in a falling off from 100% activation to zero. In contrast with the effect of other agents, the over-treated, unactivated, eggs are not injured but are readily fertilizable and develop normally. The supra-optimal exposures may then be considered essentially the same as the sub-optimal exposures, and the eggs that fail to extrude polar bodies after either treatment can be regarded as having been under-activated.

When the activated eggs were examined for cleavage, a rather surprising result was obtained. Practically none of the eggs that extruded polar bodies was found to divide, whereas most of those in which polar body formation was suppressed cleaved and developed into embryos. In the former the initial changes are very much the same as when the eggs are fertilized. The unfertilized *Urechis* egg is in the germinal vesicle stage and has a characteristic large indentation which marks the pole of the egg (Tyler, 1932*a*). The indentation reappears when the eggs are returned to normal sea water from the hypotonic solution. After exposures resulting in polar body extrusion, the egg rounds up, the membrane elevates, the germinal vesicle disappears and the polar bodies are formed in the same manner and on the same time schedule as in the fertilized egg. Still practically none of the eggs of this type divide. The other type behaves initially in a manner that might be expected for a poorly activated egg. The only change that occurs approximately on schedule is the dissolution of the germinal vesicle. The egg does not round up until after about ¾ hr. as compared with 3 min. Then the membrane is slowly elevated. Whereas the normal egg extrudes the first polar body at 30 min. and the second at 40 min. at 20° C., no sign of mitotic activity is evident in this type of egg at that time and no polar bodies appear later. In spite of their poor start most of the eggs divide, the first cleavage occurring at 80–180 min. after treatment as compared with 65 min. for the fertilized eggs.

3. *Cleavage by means of a presumptive polar spindle*

It appears then in *Urechis* that after hypotonic treatment the tendency for polar-body formation excludes that for cleavage. A similar result was noted earlier in *Cumingia* by Morris (1917) and by Heilbrunn (1925), who found after activation by heat that those treatments which gave the higher percentages of polar-body formation tended to give the lower percentages of cleavage and vice versa. Heilbrunn suggested that the first polar spindle was converted into the cleavage spindle in those eggs of *Cumingia* in which the polar bodies failed to be extruded. Evidence for this type of behaviour had been presented by Lefevre (1907) in eggs of *Thalassema* activated by acids, but the account was complicated by the occurrence of other types of behaviour, particularly submerged maturation divisions and fusion of the resulting nuclei. Convincing evidence was obtained, however, from cytological observations on *Urechis* eggs (Tyler, 1932*b*). The eggs that fail to extrude polar bodies could be readily distinguished from the others long before the time of the polar divisions. In these eggs there are formed, after the dissolution of the germinal vesicles, eighteen karyomeres (Fig. 5, IA) corresponding to the haploid chromosome number. The eggs remain in this condition during the time at which polar bodies are normally extruded. Then the karyomeres unite and later the spindle for the first division is formed. No sign of a submerged maturation division was observed and the first division figure to appear was that for the division of the egg. Furthermore, the chromosomes on the first cleavage spindle were observed to be tetrads and to be haploid (18) in number.

The division of the entire egg by means of a polar spindle was reported by Conklin (1917) in centrifuged *Crepidula* eggs, and the question has been further investigated by Morgan (1933, 1935), Morgan & Tyler (1935) and Clement (1935) in other forms. The difference between a polar spindle and a cleavage spindle appears then to be due to the position assumed in the egg rather than to intrinsic factors. In the artificially activated *Urechis* egg the "converted" polar spindle not only becomes centrally located but also assumes a position at right angles to the polar axis, so that the first cleavage plane passes through that axis as it does normally. Just what factors are responsible for the failure of the spindle in these "under-activated" eggs to approach the pole cannot as yet be stated. It appears to be correlated with a retardation in the surface changes, as illustrated by the slowness of rounding up and membrane elevation in this type of egg.

4. *Double treatment*

The *Urechis* eggs that extrude both polar bodies after the hypotonic treatment subsequently go through a series of monastral cycles in which the chromosomes divide but not the cytoplasm. Some may develop into swimmers that have "differentiated without cleavage". The polar bodies can be suppressed in this type of egg by means of a second hypotonic treatment applied during the time when they would normally be extruded (Tyler, 1932*c*). The eggs then undergo cleavage, as much as 98% dividing as compared with none for the untreated controls. If the second treatment is applied after the extrusion of the second polar body, no cleavage is obtained. This supports, then, the view that suppression of the polar bodies is essential for cleavage in this type of egg.

5. *Activation with ammoniacal sea water in* Urechis

In another species, *Urechis unicinctus*, Hiraiwa & Kawamura (1935, 1936) reported somewhat different results with eggs activated principally by means of ammoniacal sea water. With short treatment polar-body extrusion occurs normally but no cleavage follows. With longer exposures polar-body suppression occurs in many of the eggs and the submerged maturation divisions and fusion of nuclei described by many earlier investigators takes place. Either one or both polar bodies may be suppressed. Cleavage, they find, occurs regardless of the presence or absence of polar bodies. Inaba (1936) obtained similar results with eggs of the oyster *Ostrea gigas*, using again principally ammoniacal sea water for treatment, but he notes that most of the eggs that extrude both polar bodies form a monaster and fail to cleave. In neither of these investigations was there observed the type of egg in which the first polar spindle became the first cleavage amphiaster.

In view of these results an investigation of the behaviour of *Urechis caupo* eggs activated by ammoniacal sea water was undertaken by Tyler & Bauer (1937). This work has cleared up the situation to some extent. With short exposures to the ammoniacal sea water (solutions ranging from 0·002 to 0·02 molar NH_3 in sea water are effective) the previously described "poorly activated" type of egg is obtained (Fig. 5, IA). These fail to give off polar bodies and all cleave without first undergoing submerged maturation divisions. They are identical with the type obtained by under-treatment with hypotonic sea water. These occur principally after treatment that fails to activate all of the eggs and, in fact, as the percentage of total activation increases to 100% with increased exposure the percentage of eggs of this type drops to zero. This again illustrates the inverse relation between cleavage and activation previously found (Tyler, 1931*b*) with hypotonic solutions. After somewhat longer exposures, the extent of which depends on the concentration of ammonia employed, all of the eggs are activated and all extrude two polar bodies normally but none divides. Further increase in exposure time results in the failure of the first and then of the second polar body as well to be extruded in increasing percentages of the eggs.

6. *Submerged maturation divisions*

When the first polar body is extruded the second polar body invariably appears (Fig. 5, B). Those eggs that fail to extrude the first polar body form by a submerged division two nuclei and two spindles appear in the egg at the time of the second polar division (Fig. 5, C). One of three types of behaviour may follow: (1) each of these spindles may give rise to a polar body, thus producing two "second" polar bodies, and leaving two nuclei in the egg (Fig. 5, C1); (2) one spindle may extrude a polar body while the other undergoes submerged division and three nuclei remain in the egg (Fig. 5, C2); (3) both may undergo submerged divisions resulting in no polar bodies and four nuclei remain in the egg (Fig. 5, C3). These types occur in the order described upon increased exposure to the ammoniacal sea water, but with considerable overlapping. It is evident that type C1 might very well be confused with eggs that had given off both the first and second polar bodies normally, especially as in *Urechis* the first polar body normally only divides in about half of the eggs. In order to distinguish between these two types and to ascertain the cleavage behaviour of the various types, it was necessary to isolate the eggs at the time of the first polar-body extrusion and again at the time of the second. It was mentioned above that over a considerable range of exposures giving 100 % activation all of the eggs extrude both polar bodies normally but none divides. With longer exposures this type also occurs along with increasing percentages of eggs exhibiting polar-body suppression. Since there was the possibility that the longer exposure might cause the former type to divide, this type was isolated from samples of eggs that had been so treated. Of these eggs that had extruded both polar bodies normally about 14 % underwent division within 1½ hr. of the normal time. A few hours later a good many more eggs (up to 42 %) had the appearance of having cleaved, but closer examination showed that these eggs had undergone a sort of lobulation or fragmentation, giving a large number of lobules but no typical cells. Of the 14 % that had cleaved earlier, most of the eggs did not go farther than the 2-, 3- or 4-cell stage. On the other hand, when eggs that showed polar body suppression were isolated from the same samples practically all (98 %) were found to divide within 1½ hr. of the normal time and to continue cleaving and develop into embryos.

7. *Type of cleavage following polar body suppression*

The type of first cleavage exhibited by the "suppressed polar body" eggs depends upon whether two second polar bodies (type C1 in Fig. 5), one second polar body (type C2) or no polar bodies (type C3) are produced. Type C1 divides into two cells at the first cleavage, type C2 into three cells and type C3 into four cells. The typical behaviour of the various types of eggs is illustrated in Fig. 5. Since, as noted above, type C1 contains two nuclei, type C2 contains three and type C3 contains four, it is clear that the number of cells formed at the first cleavage is directly correlated with the number of nuclei present.

8. *Role of central bodies*

The result may be interpreted in terms of the central bodies as follows. When both maturation divisions occur submerged within the egg (type C3) there result, along with the four nuclei, four central bodies. These do not divide, but each gives rise to an aster and thus a tetraster spindle is formed resulting in cleavage of the egg into four cells at once. Similarly, when one second polar body is extruded (type C2), three central bodies remain and a triastral figure is formed giving cleavage into three cells at once. Likewise when two "second" polar bodies are extruded (type C1), two central bodies remain and an amphiaster is formed giving two cells at the first cleavage. When both polar bodies are extruded normally (type B), one central body remains and a monaster is formed.

In none of these different types of eggs does division of the central body occur before the time of first cleavage. If such division had occurred, the four different types of eggs would be expected to give at the first cleavage 8, 6, 4 and 2 cells respectively in the order described. The failure of the central body to undergo such a preliminary division is evidently a manifestation of its normal behaviour. In normal fertilization the central body in each of the three polar bodies and in the egg fails to divide. The formation of an amphiaster, triaster or tetraster when one, two or all three of the polar body centres are retained along with the egg centre is then consistent with their normal behaviour. But the eggs would, on this basis, not be expected to undergo more than one cleavage. The fact that cleavage continues must mean that somehow the properties of these central bodies are altered as a result of the cleavage of the egg. In the eggs that extrude both polar bodies normally cleavage fails to occur in the great majority of cases and the egg central body evidently fails to re-acquire its capacity for division. It may be suggested that the

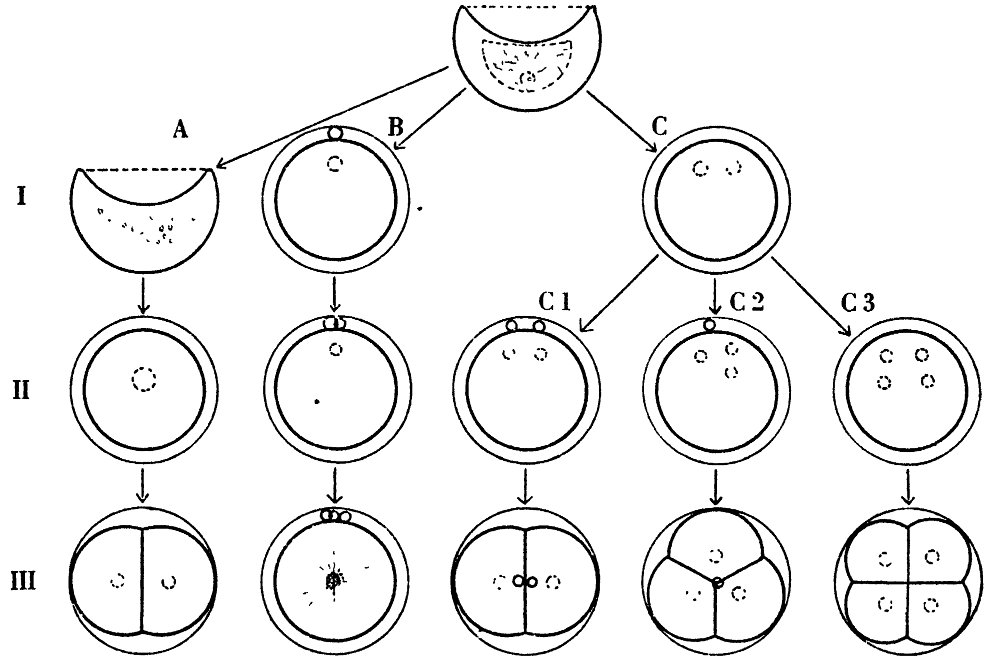

Fig. 5. Diagram of the various types of behaviour of the polar division and cleavage in *Urechis* eggs activated with ammoniacal sea water. Upper figure represents the unfertilized egg. A, B, C1, C2 and C3 represent the types obtained with increasing exposure times. With 0·01 N NH_3 in sea water at 20° C., the approximate exposures that produce the various types are: type A, ¼–1 min.; type B, 1–10 min.; type C (C1, C2, C3), 10–20 min. Row I shows the condition of the egg after the normal time of the first polar division; Row II, after the time of the second polar division; Row III, at first cleavage. The small solid circles represent extruded polar bodies; the small broken circles represent nuclei within the eggs. The eggs in III C1, III C2 and III C3 are figured in polar view, the others in side view. (After Tyler & Bauer, 1937.)

re-acquisition of ability to divide on the part of the central bodies in eggs in which polar-body suppression had occurred is due to their displacement from the centre towards the cortex of the egg as a result of the formation of spindles. Another possibility is that by becoming associated with the chromosomes in the telophase of the first cleavage, the capacity for division is re-acquired by these central bodies. These and other possibilities would be difficult to test. In favour of the former view is the failure of the central body to divide in most of the monastral eggs although the chromosomes do divide and re-constitute a nucleus. If division could be obtained in such eggs by displacing the central body toward the surface by centrifugation or some other agent, strong support would be had for the former view. Such experiments have not been reported, but possibly related are the experiments of Morgan & Tyler (1935) in which a multipolar first polar spindle is formed as a result of centrifugation.

9. *Activation of "blocked" fertilized eggs*

That the treatments resulting in 100% activation with extrusion of both polar bodies and no cleavage provides a sufficient stimulus for development was demonstrated in experiments with "blocked" fertilized eggs. Tyler & Schultz (1932) had shown that treatment of *Urechis* eggs with acidified sea water within a short time (3 min.) after insemination causes the initial developmental changes to reverse, although the spermatozoon continues its penetration into the egg. Development does not ensue upon return to ordinary sea water, although each egg contains a spermatozoon. The "blocked" eggs can be fertilized with fresh sperm and dispermic development follows. If instead they are given the kind of treatment with ammoniacal sea water that ordinarily results in 100% polar body extrusion but no cleavage, then normal cleavage and monospermic development follows (Tyler & Bauer, 1937). Other tests (Tyler & Bauer, 1937, p. 176) show the non-additive nature of the stimuli provided by simultaneous fertilization and artificial activation. It appears then that it is not because of inadequate stimulus that division fails to occur in those eggs that have extruded both polar bodies, but because of lack of part of the machinery.

10. *Inverse relation between polar body formation and cleavage*

The experiments with ammoniacal sea water and those with hypotonic solutions agree in showing that suppression of polar body formation favours cleavage in *Urechis caupo*. The failure of Hiraiwa & Kawamura (1936) to establish this for *Urechis unicinctus* appears to be due to their not having isolated the various types of eggs at the time of first polar-body extrusion. In addition to the case of *Cumingia* (Morris, 1917; Heilbrunn, 1925) and *Ostrea* (Inaba, 1936) this inverse relation has been noted in *Barnea candida* (Pasteels, 1938*a*). It is quite likely that this relation would be found to hold for all species in which the eggs are activatable before the polar bodies are formed and in which cytasters do not form. If, however, the appropriate treatment for the production of cytasters in this type of egg be found, then it would certainly be expected that the eggs would divide even after extrusion of both polar bodies.

11. *Cause of polar-body suppression*

Since polar-body suppression has been observed in practically every parthenogenesis experiment with eggs that are activatable before the first polar-body extrusion and after a variety of treatments (see Dalcq, 1928; Pasteels, 1938*a*), the effect appears to be a general result of artificial activation. Hiraiwa & Kawamura (1936) and Tyler & Bauer (1937) have shown that increase in exposure time above that necessary to give 100% activation results in suppression of the polar bodies in *Urechis* eggs activated by ammonia. Pasteels (1938*a*) obtained a similar result by increasing the concentration of the calcium solution used to activate *Barnea* eggs. With very short exposures in *Urechis* there are also obtained eggs that fail to extrude polar bodies, but these are of a different type; namely, the "poorly activated" eggs (sect. V2), in which the presumptive first polar spindle becomes the cleavage spindle.

In these the failure is evidently due to under-activation and retardation in development of the maturation spindle. In the type obtained by over-exposure to ammonia the maturation spindles form on schedule, but the divisions occur submerged within the egg. When the over-exposure is not too great only the first division is submerged and the two spindles that next form come to the surface, where each gives rise to a polar body. Slightly longer treatment results in only one of these spindles attaining the surface position, where it extrudes a polar body. Still longer exposure causes both to divide submerged within the egg. In contrast with hypotonic treatment (sect. V2), the ammonia treatment starts activation while the eggs are in the solution. The same is true for the treatment with calcium solutions employed by Pasteels (1938*a*) in *Barnea*. The failure to extrude polar bodies upon prolonged exposure is, then, attributable to an additional effect of the same treatment on the activated eggs. In fact the polar bodies can be suppressed in fertilized eggs or in eggs optimally activated by hypotonic solution by means of subsequent exposure to ammoniacal sea water (unpublished experiments). The nature of the additional effect of the prolonged treatment cannot as yet be stated. It may be a qualitatively different response on the part of the activated egg to the activating agent, or it may be an exaggeration of the original changes produced in the egg upon activation. Also, the effect may involve the physical condition of the surface, the internal movements that bring the spindle to the pole or any one of a number of other factors that may be concerned in giving the normal polar divisions. Further discussion at the present time would not be very fruitful.

It was stated above (sect. V6) that eggs that extruded the first polar body after activation with ammonia almost invariably form the second. This is to be expected from the manner of treatment. The polar body suppressing treatments are of much shorter duration than the time required for the first polar body to appear. The treatment produces a change that is inimicable to cytoplasmic division. If the first polar body appears, it means that the change had not been produced or that recovery had occurred in time and the second polar body should follow. The extrusion of one or two "second" polar bodies after a submerged first division would mean a recovery of the egg in the interim. It is obviously not possible by this manner of treatment to obtain suppression of the second polar body after normal extrusion of the first. A separate treatment between the time of first and second divisions would be required.

VI. CHROMOSOME NUMBERS

1. *Occurrence of diploid individuals*

In the absence of the spermatozoon which normally restores diploidy by contributing its haploid set of chromosomes to the egg, one might expect the artificially activated egg to develop invariably into a haploid individual. However, the early cytological investigations showed that this was not the case, but that diploid and other chromosome numbers were also obtained. In fact the only individuals that have been reared to or near the adult condition have turned out to be diploid (Paramenter, 1920, 1925, 1933; Goldschmidt, 1920). The haploid individuals generally die in an embryonic stage. This non-viability of artificially produced haploid animals has been the subject of many investigations (see Fankhauser, 1937*b*, 1938*a*; Kaylor, 1940), but a discussion of the question is beyond the scope of the present review. Of more immediate interest is the question as to how the artificially activated egg avoids haploidy and the resulting early death. This has been investigated in recent years principally in eggs of the frog and to some extent in eggs of the silkworm and *Urechis*.

2. *Regulation in* Urechis

In a cytological investigation (Tyler, 1932*b*) of *Urechis* eggs activated by hypotony, the normal embryos were found to have the diploid chromosome number. This is perhaps not surprising, since the embryos develop from eggs that fail to extrude polar bodies. In this particular type the presumptive first polar spindle becomes the first cleavage spindle (see sect. V3). The first cleavage spindle shows the haploid number of tetrads. The first division of the egg is evidently a reduction division and each blastomere is found to have the haploid number of dyads. If the next division were also reductional, then each of the resulting four cells would be expected to have the haploid chromosome number. Counts made at anaphase of the second division showed, however, the diploid number. In anaphase of the third and the fourth cleavage diploid eggs were again found, and also some exceptional haploids.

It is evident that the mere utilization of the maturation divisions for cleavage is not sufficient to insure regulation to diploidy. It is attained by the occurrence, in most of the eggs, of an extra division of the chromosomes without cytoplasmic division. The cause of this behaviour is unknown. It may perhaps be due to the fact that in normal maturation the second polar division follows very rapidly after the first without the chromosomes forming a resting nucleus in the interim, while here the interval between first and second division is longer (approximately that of normal cleavage) and the chromosomes enter the resting stage in the interim. Whatever may be the true explanation, it may be noted that this extra division of the chromosomes is consistent with the behaviour of the chromosomes in eggs of many animals (cladocerans, ostracods, aphids, rotifers, etc., see Vandel, 1931) that undergo natural diploid parthenogenesis. In those eggs, one polar body is extruded and the second spindle becomes the first cleavage spindle. For this first division an extra division of the chromosomes occurs, whereas this would not have happened if a second polar division took place. A still more closely analogous behaviour was reported by Doncaster (1911) in the case of diploid parthenogenesis in the gall-fly, *Neuroterus*, in which both polar divisions are omitted.

The types of polar body suppression obtained in *Urechis* eggs activated by ammonia (see sect. V6) involve submerged maturation divisions and fusion of the resulting nuclei. While no detailed cytological study has been made, the occurrence of diploid embryos (noted by Hiraiwa & Kawamura, 1935) is not too difficult to interpret. Type C1, which extrudes two "second" polar bodies, is essentially the equivalent of normal fertilization, the haploid egg pronucleus fusing with a haploid polar body nucleus instead of with the sperm pronucleus. This gives then a diploid nucleus for the first division and diploid development should follow. Type C2, which extrudes one second polar body, is the equivalent of a dispermic egg and type C3, which extrudes no polar bodies, is the equivalent of a trispermic egg. Since these divide into three and four cells respectively at the first division (sect. V7), each cell should have the diploid constitution unless there are irregularities in the distribution of chromosomes on the triaster or tetraster.

In *Urechis*, the eggs that extrude both polar bodies fail to divide as a rule. Since upon suppression of the polar bodies either the utilization of the first polar division for cleavage or the occurrence of submerged divisions leads to diploidy, there is not much opportunity for haploid development. From such information that is available in other species, it may be surmised that this situation is probably the same in most marine invertebrates aside from the sea-urchin. In eggs of the frog and insects other methods of regulation have been shown to occur.

3. *Regulation in the frog*

Following the original finding by Parmenter (1920) and Goldschmidt (1920) that metamorphosed frogs, produced by the puncture method of activation, were diploid, a number of investigators have attempted to determine the manner of regulation. In recent years Parmenter (1933, 1940) and Kawamura (1939*a*) have made extensive studies of the problem and have uncovered at the same time larvae with a variety of other chromosome numbers besides haploid and diploid.

Out of 29 parthenogenetically produced embryos and adults of *Rana pipiens* and *R. palustris* Parmenter (1933) found 12 haploids, 10 diploids, 1 triploid, 4 haploid-diploid, 1 diploid-triploid and 1 diploid-triploid-tetraploid. He noted that, whereas in all the haploids that were examined the first cleavage was undelayed, in the diploids a delay of approximately one cleavage period occurred in some cases and no delay in others. He points out that diploidy in the frog may arise by more than one method. Where a delay in the time of first cleavage occurs, the chromosomes may double either by a monastral division followed by an amphiaster (see Wilson 1925,

p. 168, for example) or by an anastral division (see Bataillon & Tchou-Su, 1930; Tchou-Su, 1931) followed by an amphiaster. The latter seems more likely, since there is as yet no evidence that in artificial parthenogenesis a dicentric division can follow a monaster. Where no delay in the first cleavage occurs, he suggests that the diploidy may be brought about by one of the following means:

(1) Retention of second polar body.
(2) Omission of polar divisions.
(3) Diploidy in virgin eggs.
(4) Progressive regulation during cleavage.

Retention of the polar body has been described in anuran eggs activated by puncture (Bataillon & Tchou-Su, 1929) and by fertilization with foreign sperm (Tchou-Su, 1931); also in inseminated over-ripe eggs (Bataillon & Tchou-Su, 1934). It has been described in many cases of natural parthenogenesis (see Vandel, 1931). But direct observations on polar-body extrusion in the living egg (see below) have not as yet shown the production of diploid parthenogenetic frog embryos by this method.

The second method is that in which the presumptive polar spindle becomes the first cleavage spindle (Tyler, 1932*b*; sect. VI 2). Since in the frog's egg the first polar body has been extruded before activation, it is the second polar division that would be involved rather than the first as in *Urechis*. This method would then be the same as that found in most cases of natural diploid parthenogenesis. However, observations on polar-body extrusion alone would not suffice to distinguish this from the first method.

The third should not be considered a method of regulation if the starting-point is a tetraploid female. In the frog, Bataillon & Tchou-Su (1929) described a diploid polar division in one out of thirty eggs examined. This probably arose from retention of the first polar nucleus or failure of synapsis rather than from initial tetraploidy. In any event the occurrence of such virgin eggs would furnish a starting-point for diploidy without cleavage delay. While there is not much information concerning the natural occurrence of polyploids in anurans, in urodeles Fankhauser (1939) finds spontaneous triploidy to the extent of 1% in *Triturus viridescens* and 10% in *Eurycea bislineata*.

That the fourth type, progressive regulation, occurs is evident from those cases described by Parmenter in which different numbers occur in different cells of the same tadpole. In particular, the occurrence of tadpoles in which only a few diploid cells are found among the haploids shows that the doubling can take place at a late stage. Such regulation during segmentation has been described in natural parthenogenesis in one of the butterflies, *Solenobia triquetrella* (Seiler, 1923, 1939), in grasshoppers (King & Slifer, 1934) and in the phasmids (Cappe de Baillon, Favrelle & de Vichet, 1938).

Parmenter's findings of the occurrence of a delay in the first cleavage in the diploids and no delay in the haploids have been confirmed and extended in *Rana nigromaculata* by Kawamura (1939*a*), who has examined the chromosome numbers and cleavage history of 413 individuals and has described some new karyotypes among them. Activation was by the method of puncturing blood-smeared eggs with a fine needle. None of the control eggs developed. Out of about 164,000 eggs that were pricked, 569 larvae (0·35 %) of the hatching stage were obtained. Twelve of these metamorphosed and three died at metamorphosis. Out of 1073 eggs whose cleavages were observed, 200 (19 %) showed a delay of one period or more. In about half of these the karyotypes were later determined. A summary of the data is given in Table 1. Practically all of the haploids show an undelayed cleavage and most of the diploids were delayed. The origin of the latter is accountable, then, on the basis of the delay as discussed above. For the four undelayed diploids it cannot be decided which of the four mechanisms listed above supplies the regulation. The origin of the triploids is difficult to interpret. They are unexpectedly frequent (23 %) and practically all show undelayed cleavage. Kawamura suggests that they arise from eggs that had undergone a submerged first maturation division (presumably before activation) and then extruded one second polar body as in the case of *Urechis*. However, it should be noted that if the first cleavage of such an egg produces three cells, as in *Urechis*, only diploidy would be expected. Very likely this does not occur, or it would have been seen in the examination of the eggs for the determination of cleavage time. This difference between the frog and *Urechis* would be quite consistent with the difference in origin of the first division figure. In the frog the amphiaster evidently arises from one of the cytasters (sect. III 2), while in *Urechis* an amphiaster, triaster or tetraster is formed by the association of the egg central body with one, two or three submerged polar-body central bodies (sect. V 8). Submerged polar divisions would not then be expected to affect the formation of a dicentric first cleavage figure in the frog and this, in view of the undelayed cleavage, seems to be a reasonable interpretation for the origin of the triploids. Polar-body histories and also cytological observations on fertilized controls, which would be desirable in view of the high incidence of triploidy, are unfortunately lacking in Kawamura's excellent investigation. This lack also makes the origin of most of the

Table 1. *Types of individuals obtained by artificial activation in* Rana nigromaculata *(from Kawamura, 1939a)*

Karyotype	Number of individuals	Number with known cleavage history	
		Undelayed	Delayed
Haploid	202 (49 %)	197	1
Diploid	86 (21 %)	4	76
Triploid	95 (23 %)	89	1
Tetraploid	4 (1 %)	1	3
Hexaploid	12 (3 %)	2	9
Hyperploid	1 (¼ %)	0	1
Haploid-diploid	8 (2 %)	8	0
Haploid-triploid	3 (¾ %)	3	0
Haploid-pentaploid	1 (¼ %)	1	0
Triploid-hexaploid	1 (¼ %)	1	0

other karyotypes ambiguous. It is clear, however, from these data, too, that more than one method of regulation may occur in artificially activated eggs of the frog.

Parmenter (1940) attempted the difficult task of determining the polar-body history in the artificially activated eggs. All but thirteen of 559 pricked eggs of *Rana fusca* showed both polar bodies. In two of the eggs the single polar body had the appearance of the second. Of the thirteen eggs with one polar body only one developed, but died as a gastrula. 7 % of the others cleaved, and from these there were obtained eleven embryos whose karyotypes were determined. These were

3 pure haploids,
4 haploid-diploids,
1 pure diploid,
3 diploid-triploids.

None of these showed a delay in early divisions. Since they had also all given off both polar bodies, the increase in chromosome number above the haploid must have occurred in later cleavage or embryonic stages. It may seem unlikely that all the cells in a late stage should undergo regulation, and Parmenter suggests that one or more cells in the pure diploid might be haploid. On the other hand, there is at present no good reason to expect regulation in one cell of an embryo and not in another. For that matter there is no evident reason for expecting regulation at all in the late stages, especially since the spindle with the haploid nucleus is fully able to divide the cytoplasm in the large cells of the early cleavages.

The data on these various species of frogs show that regulation to diploidy occurs principally, but not exclusively, by a delay in cleavage. Generally the delay consists of one period in the first division (68, possibly 77 out of 91 diploids). Occasionally (two cases) the delay is in the second or third divisions. Of the twenty-one metamorphosed animals and twenty-four nearly metamorphosed, which are all diploid, sixteen had shown delayed first cleavage and three had not. The manner of regulation has, then, no particular influence on the ability to develop. This point was exemplified in *Urechis* too.

4. *Regulation in the silkworm*

The cytology of artificially activated eggs of *Bombyx mori* has been studied by Sato (1931), Bataillon & Tchou-Su (1933) and Frolowa (1935). All agree that both polar divisions occur. According to Sato the haploid egg nucleus divides and the two daughter nuclei fuse to give a diploid segmentation nucleus, which gives diploid development from the start. According to Bataillon & Tchou-Su, segmentation can begin and continue for some time with haploid nuclei. Occasionally a germ band is formed, but the haploid development never goes as far as hatching. Frolowa found, in material supplied by Koltzoff (1932), two types of behaviour: (1) doubling of the chromosomes at the first cleavage, (2) doubling at the late division stage when the nuclei migrate to the surface. The doubling occurs by union of nuclei that have remained close to one another after division. In type (1) the egg nucleus is found close to the surface near the micropyle; in type (2) it migrates deep in the egg opposite the micropyle. Type (1) is the less frequent and eggs of this type were found to be greatly retarded in their development, often going no further than the first cleavage. In type (2) the doubling-up does not occur simultaneously in all the nuclei, and blastoderms are found with haploid as well as diploid nuclei. Bi- and multi-nucleate cells are also found. Quite normal-looking post-diapause embryos were seen to have variable chromosome numbers, so Frolowa concludes they must have arisen from type (2) eggs.

In the silkworm, then, the principal method of regulation appears to be that of progressive fusion of nuclei as described in *Solenobia*, grasshoppers and phasmids (sect. VI 4). That other types of regulation may occur is indicated in eggs that Koltzoff activated with iodine and subjected to low temperature. These showed fusion of the egg nucleus with polar-body nuclei. The method of treatment is undoubtedly important in determining the method of chromosome regulation.

VII. SEX

1. *Theoretical*

The sex of individuals obtained by artificial activation will depend, in most animals, upon the history of the chromosomes, since, as is well known, they carry the sex-determining factors. On the basis of the genic balance concept (Bridges, 1925, 1939), maleness or femaleness is determined by the ratio of the number of sex-chromosomes to the number of sets of autosomes. Since there are two main types of sex determination in animals, the male digametic (*Drosophila*, mammals, etc.) and the female digametic (Lepidoptera, birds, etc.), the expectation after artificial parthenogenesis will depend, too, on the particular species involved. In the male digametic type, the autosomes (A) contain a preponderance of male-determining genes and the sex-chromosomes (X) a preponderance of female-determining. Where the ratio of $X : A$ is one or greater the result is female; where it is one-half or less the result is male. In the female digametic type the situation is reversed.

Artificial parthenogenesis in the male digametic type should, then, always result in females, regardless of possible increase in karyotype and method of increase (polar-body suppression or fusion of segmentation nuclei), provided whole chromosome groups are involved. In the female digametic type the possibilities are more varied. Thus, there are two kinds of reduced eggs, one containing a sex-chromosome (termed Z, in this type) and one without it. The former (ZA) should give a male regardless of whether or not it later becomes diploid or polyploid. The latter should be expected to give a "hyperfemale", regardless again of whether or not it increases its karyotype, but such individuals with no sex-chromosomes would not be expected to develop very far (see Poulson, 1940). If fusion of the egg nucleus with one or more polar-body nuclei occurs, males or females would be expected, depending on which polar division was reductional for the sex-chromosome and which nuclei fused. If conversion of the first polar division into a segmentation division occurs along with sex-chromosome segregation at that division, then mosaic individuals composed partly of male and partly of "hyperfemale" cells would be expected.

Another feature of the female digametic types is the occurrence in many species of two visibly distinct kinds of eggs which even before the maturation divisions have occurred are determined to produce upon fertilization males and females respectively (see Vandel, 1939). Since the sex of the individuals conforms, nevertheless, to the chromosome constitution, the manner of segregation of the sex-chromosome (whether or not it remains in the egg nucleus) evidently is determined in those species by the egg-cytoplasm.

There are relatively few cases in which the artificially activated eggs have been reared to the stage of visible sex differentiation. These occur principally in the frog and the silkworm.

2. *Sex of parthenogenetic frogs*

The early data of Loeb (1921) and Parmenter (1925) showed in *Rana pipiens* a preponderance of thirty-two males to twenty-three females. Since the frog evidently belongs to the male digametic type (see Witschi, 1939), the occurrence of males after artificial activation is difficult to explain. Parmenter suggested that it may be due to over-ripeness of the eggs which, as R. Hertwig (1921) and Witschi (1924) showed, leads to the production of males. He also considered the possibility that the frogs used might belong to an undifferentiated race (see Witschi, 1939) in which external factors, such as high temperature, may transform genetic females into males.

More recently Kawamura (1939*b*) employed females of well-differentiated races of *Rana nigromaculata* and *R. japonica* and took precautions to avoid over-ripeness. He obtained, from the artificially activated eggs, eighteen females, two animals with mullerian ducts but whose gonads were transforming into testes, two with rudimentary testes and one somewhat abnormal male. The preponderance of females and absence of typical males agrees well with the expectation on the basis of male digamety in the frog. As to the exceptions, Kawamura suggests that the abnormal development of other organs might cause degeneration of the ovaries, or, less probably, that some over-ripeness had occurred. Another possibility is that exceptional males arise by sex-chromosome elimination such as occurs occasionally in *Drosophila* (Morgan & Bridges, 1919) and regularly in *Sciara* (Metz, 1938).

Kawamura (1940) has also examined the gonads of triploids obtained by artificial activation. He finds that the germ cells degenerate early, starting at the genital ridge stage, and concludes that triploidy may be incompatible with the development of normal ovaries in the frog.

3. *Sex of parthenogenetic silkworms*

Data on the sex of silkworms obtained by artificial parthenogenesis are presented by Sato (1931) and by Astaurov (1936). Sato obtained mostly males from eggs activated with hydrochloric acid. Since the silkworm belongs to the female digametic group of animals, and since Sato reported that development starts with the haploid egg nucleus which regulates to diploid after the first division (see VI4), then the males presumably arise from those cases where the egg nucleus contains the *Z* chromosome. Those cases in which the egg nucleus lacked the *Z* chromosome would presumably die in an early embryonic stage. Sato does not account for the occurrence of females in his material. Astaurov, on the other hand, obtained almost exclusively females (25,759 females to 11 males) from eggs activated by heat (plus hydrochloric acid in some cases to eliminate diapause). Genetic tests of 143 of the females showed 141 to be heterozygous for all the autosomal factors which had been present in their heterozygous mothers, while two were homozygous for one of the recessive genes. Although cytological studies on this material have not as yet been reported, the great preponderance of heterozygous females evidently means that the regulation occurs either by fusion of the egg nucleus with one of the polar-body nuclei containing the complementary (non-identical) chromosome set or by omission of the reduction division. Astaurov also reported the occurrence of many tetraploid, heterozygous, females. These may well have arisen from the fusion of all three polar-body nuclei with the egg nucleus or omission of both meiotic divisions.

From Sato's and Astaurov's results it appears, then, that the sex obtained will depend upon the method of activation employed (perhaps also on the race of silkworm used) and consequent method of regulation to diploidy.

4. *Other animals*

Apart from the old observations of Delage (1909) on the sea-urchin, the only other case of artificial parthenogenesis in which the sex of the offspring is reported is that of the rabbit (Pincus, 1939*b*; Pincus & Shapiro, 1940). Delage had obtained two animals that lived long enough to be identified as males. Since the sea-urchin evidently (see Wilson, 1925, p. 758) belongs to the male digametic group, this result is not readily interpretable on any simple basis.

The seven rabbits which were reported as being unquestionably parthenogenetic were all females. Since the rabbit belongs to the male digametic group, the production of only females is to be expected. Pincus (1939*a*) has described haploid, diploid and tetraploid chromosome numbers in the early cleavages of artificially activated eggs, but it is quite likely that these seven rabbits are all diploid. One of them, tested genetically (Pincus, 1939*b*), turned out to be heterozygous for a coat character. This means then that diploidy was attained here by fusion of the egg nucleus with a polar-body nucleus. Since the first polar body has already been extruded at the time of activation, it is evidently submergence of the second division that is involved.

VIII. METHODS AND THEORIES OF ACTIVATION

1. *General remarks*

As is well known from the earlier work, a wide variety of physical and chemical agents are capable of activating animal eggs. The investigations of the last decade have not added much that is essentially new in regard to methods, nor does that appear to have been their intention. There were already many more methods than could be reduced to a common factor. Neither have they uncovered any correlations between the effective treatments for eggs of various species and properties or characteristics of the eggs. While eggs of one species of animal may be activated by a number of different agents, those of another species may respond to a different set. Eggs of a large number of different species will respond to the same agent (e.g. heat, acids), but no attempt has yet been made to determine in such cases the basis for differences in amount of treatment required. Whether or not there is a single agent that is capable of activating the eggs of any (all) species of animals cannot as yet be decided. Since there are a wide variety of possibilities in regard to manner of application, concentration (or intensity) and time of exposure, negative results with a particular agent are not of very much significance and, probably for that reason, are not very often reported in the literature.

It is clear that there is very little specificity in regard to activating agents. Thus eggs of the sea-urchin can be activated by such diverse agents as puncture, heat, cold, ultra-violet radiation, radium emanations, acids, bases, isotonic salt (some of the constituents of sea water) solutions, hypertonic and hypotonic solutions, fat solvents and some alkaloids. This contrasts strikingly with the high degree of specificity in fertilization. One might suppose that, in fertilization, a specific initial reaction between the egg and sperm releases a non-specific activating agent, but there is no experimental evidence for this view. What the common factor may be in the various parthenogenetic agents and the sperm has remained obscure. The recent work, while not contributing much to the questions raised here, has advanced to some extent our knowledge as to the mechanism of activation. It has, in general, been confined to the investigation of the conditions of action of a few kinds of activating agents. These have been principally radiation, acids, heat and unbalanced salt solutions. There has also been a renewed interest in sperm extracts and modified sperm.

2. *Sperm extracts and modified sperm*

There have been many attempts to extract the activating principle from sperm, but a survey of the literature (see Loeb, 1913; Sampson, 1922; Just, 1930; Godlewski, 1934) reveals no well-substantiated positive result. The most recent attempt is that by Frank (1939), who obtained negative results with an extract prepared by heating sea-urchin sperm in sea water or salt solutions. He did obtain, however, in his extracts a substance that reacted with the sperm agglutinating substance (Lillie's fertilizin) obtained from eggs. In the keyhole-limpet, Tyler (1939) independently obtained an anti-sperm agglutinin (anti-fertilizin) by extracting frozen and thawed sperm with sea water or salt solutions. The keyhole-limpet extracts were found to contain, in addition, a lysin that specifically dissolves the membrane that surrounds the egg in this species. Anti-fertilizin has also been obtained from sea-urchin sperm by Southwick (1939) and by Hartmann, Schartau & Wallenfels (1940).

Fertilizin was considered by Lillie (1919) to play a central role in the activation of the egg (see Just, 1930, for most recent review). The agglutinating action of this substance on spermatozoa has been studied recently by Tyler (1940*a*). Investigations of its chemical properties (Tyler & Fox, 1940) show it to be a protein. Lillie considered activation to involve a reaction between fertilizin and an anti-fertilizin contained within the egg. Tyler (1940*b*) has succeeded in extracting, after removal of fertilizin, an anti-fertilizin from sea-urchin eggs, and to that extent, then, contributes some support to that part of Lillie's views. Aside from its possible bearing on fertilization this finding is of interest in that it demonstrates the presence of a subsurface substance that is complementary (acts as an antibody) to the surface substance. The exact role of these various substances in the activation process remains yet to be determined.

Separation of the activating and genetic functions of the sperm was first accomplished in the classical experiments of O. Hertwig (1911), by exposure of frog spermatozoa to radium emanations. Appropriately prolonged exposure completely damages the chromatin so that it takes no part in development, although the ability of the sperm to activate eggs is unimpaired. This gives, then, an essentially parthenogenetic (gynogenetic) development. Similar experiments were performed on salamanders (O. Hertwig, 1913), fish (P. Hertwig, 1916; Oppermann, 1913), sea-urchins (G. Hertwig, 1912) and on annelids (Packard, 1914, 1918). Exposure of frog sperm to ultra-violet radiation, X-rays or trypaflavin (Dalcq, 1931; Dalcq & Simon, 1931; Rugh, 1939) has been shown to give similar results. Rugh & Exner (1940) have also confirmed an early experiment by G. Hertwig (1913) in which eggs of one species are inseminated with irradiated sperm of another species of anuran. The control hybrid generally dies before gastrulation, whereas the "hybrid" with irradiated sperm develops considerably further (hatching and beyond). In the silkworm attempts by Astaurov (1937) to get parthenogenetic (gynogenetic) development by this means have not proven very successful, since doses that would be strong enough to inactivate the sperm chromatin completely are injurious to the males. The same difficulty might also be encountered in mammals unless ejaculated sperm and artificial insemination were employed. Complete androgenetic development, on the other hand, was more readily obtained by Astaurov by inseminating irradiated silkworm eggs with normal sperm. The offspring were all male, as is to be expected in this form. A discussion of this and other work on androgenesis is beyond the scope of the present review.

3. *Superficial cytolysis*

Loeb (1913) proposed the view that all activating agents work by producing a superficial cytolysis along with an increased respiration in the egg. To protect the egg against complete cytolysis a second "corrective" factor such as hypertonic treatment was thought to be essential. This theory has been criticized both on the basis of the drop in respiratory rate exhibited by eggs of various species upon fertilization (Whitaker, 1933) and on the basis of the effectiveness of a single treatment (Just, 1922, 1930).

Recently Lindahl (1938*a*) has reported some experiments which he considers as supporting Loeb's views. Loeb had supposed that the spermatozoon brought both the lytic and corrective factors into the egg, but there was no experimental demonstration that the factors could be separated so as to cause cytolysis of the egg by homologous sperm. Lindahl supplies such evidence by fertilizing, with normal sperm, sea-urchin eggs that had been treated for some time with sodium thiocyanate, carbon monoxide and other agents. Upon fertilization such eggs may undergo cytolysis completely or locally at the entrance point of the sperm. The result is taken to mean that the treatment causes an increased acid-formation (Runnström, 1933, 1935 *b*) or (and) a weakened stability of the plasma-colloids (Runnström, 1928) so that the stimulus of the sperm results in cytolysis. This might, then, imply that the sperm introduces a lytic agent which in the weakened egg has a more profound effect than in the normal, but it certainly does not reveal a corrective factor.

It is true that most activating agents if applied sufficiently long result in cytolysis, although there are exceptions such as certain degrees of hypotony, acidity, etc. (Tyler, 1931 *b*; Pasteels, 1938 *b*). Designation of the change normally produced by fertilization or artificial activation as incipient cytolysis implies a similarity in the two processes. But cytolysis is as yet a rather poorly defined process so that it does not involve much more than the use (or abuse) of terminology when the activation reaction is termed cytolytic. Analogously, one might consider as cytolytic the process by which a nerve conducts an impulse because over-stimulation results in destruction. Here again nothing seems to be gained by the use of the term. In both cases it remains more essential to have an account of the process in better defined physical and chemical terms (see sect. VIII 7).

4. *Radiation*

When a physiological effect can be produced by radiation, a determination of the wave-length-effectiveness relation will further the analysis. Such a determination in the case of artificial activation has recently been made by Hollaender (1938), who for the first time employed *monochromatic* ultra-violet light. Lillie & Baskerville (1922) had shown that sea-urchin eggs could be activated by ultra-violet rays. Hollaender finds that the shorter wave-lengths are the more effective. He determined the wave-length dependence of activation and found it to resemble the inactivation curve of a protein such as urease or tobacco mosaic virus. Nucleic acids, which have their maximum absorption in longer wave-lengths, do not appear to be involved in the initial activation mechanism, but many organic compounds besides proteins show increased absorption at the short wave-lengths. While definite conclusions cannot as yet be drawn, it may be noted that a denaturation-like action of the ultra-violet (see Clark, 1936) on a protein constituent of the egg might be consistent with the calcium-release theory discussed below (sect. VIII 6).

Harvey & Hollaender (1938) also activated non-nucleated as well as nucleated fragments of sea-urchin eggs with ultra-violet rays. They conclude that the radiation affects primarily the cytoplasm. Another method which offers interesting possibilities is that of localized irradiation with a narrow beam of ultra-violet, by which Tschakotine (1935) has succeeded in activating sea-urchin and pholade eggs. Other parthenogenesis experiments with radiation will be discussed below (sect. VIII 6) in connexion with the role of calcium.

5. *Role of acidity and hypertonicity*

As a result of an extensive series of investigations on the artificial activation of starfish eggs, R. S. Lillie (1934) has developed what may be termed a bimolecular theory of activation. According to this hypothesis an activating substance (*A*) is formed in the egg as a result of the chemical union of a hydrolytic product (*B*) and a synthetic product (*S*). Substance *B* is considered to be produced in the egg by the action of acid, while *S* is formed (or increased in quantity) by hypertonicity or other water-abstracting agents. Since a review by Lillie is scheduled to appear in *Physiological Zoology*, only a brief account of the details and experimental basis of the theory will be given here.

Lillie (1926, 1927) showed that weak (penetrating) acids readily activated starfish eggs, while bases were ineffective. The different weak acids were found to give comparable activation at nearly the same *p*H (*ca.* 3·8). By use of the common ion effect he showed that the effectiveness of a given acid was dependent on the concentration of the undissociated molecules present in the solution. It would be of interest here to determine the active form within the egg by the method used by Tyler & Horowitz (1938 *b*) for the respiratory stimulating and cleavage blocking action of the substituted phenols. While data is not available for that kind of analysis, Lillie's results show that the effectiveness of the weak acids depends on the ready penetration of the undissociated form and is thus in accord with the general results of permeability studies (see Jacobs, 1939).

A comparison of the action of heat (Lillie, 1931) with that of acid brought out many similarities. Both showed similar time relations for partial and complete activation, were completely effective only in the pre-maturational period of the egg, were capable of completing a partial activation by one of them and were active in absence of oxygen or presence of cyanide. Since heat above the physiological range is known to produce acid (e.g. lactic in heat rigor) the activating effect is interpreted as due to the production of acid in the egg. Hypertonic sea water was found to be much less effective than heat or acids as an activating agent for starfish eggs. It could, however, be used to supplement and complete a partial activation with heat or acids. In contrast to the latter agents, hypertonicity would not act in absence of oxygen or presence of cyanide. Lillie also found that keeping untreated (or partially activated) eggs in anaerobic conditions considerably prolonged the period during which they could be activated (or activation completed) by heat, acid or hypertony. In addition, such exposure increased the susceptibility (decreased treatment-time) of the eggs to activation by heat or acid. He interprets this as due to the accumulation under anaerobic conditions of an amount of acid that is not sufficient to cause activation but which will shorten the subsequent acid treatment required. To account for the fact that activation can be obtained by hypertony as well as acidity he assumes that the essential activating substance (*A*) results from the combination of two substances (*B* and *S*) which may be initially present in low concentration. Acidity produces *B* by hydrolysis of some precursor (phosphagen was suggested), while hypertonicity produces *S* (perhaps a specific protein) by dehydrolytic synthesis. Thus, according to the mass action law, increase in the concentration of either B or S would lead to the formation of A, and this would explain activation by either acidity or hypertonicity alone or in combination.

Runnström (1932) has pointed out that this theory is partly in accord with his own views and experiments. He had shown that an acid is produced upon fertilization and upon treatment with activating agents such as heat; also that acid is formed in unfertilized eggs in anaerobic conditions. But his finding that hypertony, too, leads to acid formation is not in as good accord. Runnström's view is that, upon activation, there must be a coupling between the respiration and a reaction that restores the original substance so that the formed acid disappears. Where the coupling fails, cytolysis would result. The acid that is formed has not as yet been identified chemically, but it is evidently not lactic; nor does it appear to be derived from a dephosphorylation or a catheptic proteolysis (Runnström, 1935*b*).

Lillie (1934) has examined his hypothesis on the basis of the kinetics of a bimolecular reaction, by determining the relation between various preliminary (partially activating or sensitizing) treatments with hypertonic sea water and the subsequent treatment with acid required for complete activation. His data are shown to fit the theoretical curve rate of a bimolecular reaction (disregarding reverse reaction) and thus support his theory. Discordant results were obtained, however, with hypotonic sea water. A preliminary exposure to this agent, he expected, would increase the subsequent activation-time in acid solution, but instead a decrease was usually obtained. He suggests that the hypotony may have some activating (acid-producing) effect which may compensate or mask the influence of dilution on the S-producing reaction.

This highly interesting theory is quite consistent in the main with other developments in the field, such as the role of calcium (sect. VIII 6) and the presence of complementary substances (sect. VIII 2). It is to be hoped that further work will lead to more specific hypotheses and reveal the exact nature of the postulated substances; also that the objections raised by Dalcq, Pasteels & Brachet (1936) on the basis of Lillie's failure to determine activation percentages at early stages and the possibility of superactivation will be taken into account.

6. *Role of calcium*

The work of two separate laboratories, Dalcq's in Belgium and Heilbrunn's in America, has converged recently in emphasizing the importance of calcium in activation.

Dalcq (1928) and Pasteels (1935, 1938*a* and *b*) have examined in many of their own experiments and those of other investigators the effectiveness of unbalanced isotonic salt solutions in activating the eggs of a number of invertebrates. They find that pure calcium chloride solutions or calcium-rich solutions will activate the eggs of all the animals investigated in this regard. This includes the polychaetes, *Pomatoceros* and *Hydroides*; the gephyreans, *Phascolion* and *Thalassema*; the mollusc, *Barnea*; and the echinoderm, *Asterias*. In *Barnea* and *Hydroides*, potassium chloride also shows some activity, but this appears to be correlated with the degree of maturity of the egg, which Pasteels (1938 *a*) interprets as the quantity of calcium with which it is charged while in the ovary. On examining the action of various parthenogenetic agents in the presence of various combinations of isotonic salt solutions, Pasteels finds them to be generally ineffective in solutions lacking calcium. Thus, in *Barnea candida*, activating treatments with butyric acid, isotonic sodium chloride, ether, acetone, hypertony, isotonic sodium citrate, heat or ultra-violet rays are all ineffective when applied to eggs immersed in calcium-free salt solution. In the case of three agents, potassium chloride, alkalinity and hypotonicity, mere immersion in calcium-free solution is not sufficient to prevent activation, but a prior treatment with citrate renders the eggs unresponsive to these agents. They are not injured by the treatment but rather sensitized, since a trace of calcium chloride will subsequently activate all the eggs. Simon (1939) reports similar results for the activation of *Barnea* eggs with radium and correlates them with effects of the radiation on permeability.

This dependence on the presence of calcium for activation was shown considerably earlier by Heilbrunn & Young (1930) in experiments with ultra-violet rays on *Arbacia* eggs. Eggs that were treated with oxalate to remove calcium (presumably from the cortex) failed to respond to activating doses of ultra-violet, but recovered their activability when calcium was restored. Heilbrunn & Wilbur (1937) extended this work to *Nereis* and found that after removal of calcium by means of citrate the eggs failed to respond to activating treatment with ultra-violet, isotonic sodium chloride or potassium chloride. They noted, too, that the eggs became sensitive to calcium chloride solutions and, to a lesser extent, to sea water, after immersion in the citrate solutions. Lindahl (1938 *b*), citing experiments on inhibition of fertilization by acid (Tyler & Scheer, 1937), suggests the results with citrate might be due to acidity. While it seems unlikely that *p*H was not taken into account, it would nevertheless be of interest to control it by use of a suitable buffer such as glycylglycine (Tyler & Horowitz, 1937).

The fact that three of the principal cations of sea water favour (directly or indirectly) activation and that activation does not occur spontaneously in sea water (except occasionally in certain species) suggested that the fourth cation, magnesium, was inhibitory. This was indeed found to be the case by Wilbur (1939), who was able to inhibit reversibly the response of *Nereis* eggs to ultra-violet by immersion in magnesium-rich sea water. Another kind of experiment that may be correlated with these findings is the "reversal" of fertilization in *Urechis* (Tyler & Schultz, 1932) by means of citrate as well as acid sea water.

Pasteels considers calcium to be the true activating agent, while other agents act through the intermediation of calcium either in the external or internal medium of the egg. The other agents are, then, sensitizers. These he classifies in two groups: (1) acids, sodium chloride, citrate, etc., which act only as a function of external calcium, are termed external sensitizers; (2) potassium chloride, alkali, hypotony, which act in absence of external calcium but not on citrated eggs, are termed internal sensitizers. A sensitizer, he believes, may act by increasing the permeability (in some cases producing a permeability) to calcium (see also Simon, 1939), or, in a manner suggested by Heilbrunn, by changing the equilibrium between free and combined calcium. Pasteels also considers the action of lack of calcium in prolonging the life span of unfertilized eggs (Schechter, 1937) as consistent with the stimulating role of calcium. However, there are other agents that may increase the life span of unfertilized eggs (see Tyler, Ricci & Horowitz, 1938; Tyler & Dessel, 1939) that do not appear to involve lack of calcium. This weakens somewhat the argument from that direction.

Heilbrunn's theory of activation is essentially his calcium-release theory for stimulation in general (see Heilbrunn, 1937, chap. 37). The stimulating agent is considered to release calcium from a calcium proteinate gel in the cell cortex and the free calcium is then supposed to initiate a protoplasmic clotting somewhat like blood clotting. Evidence that ultra-violet can cause such a breakdown of a calcium proteinate gel is reviewed by Heilbrunn & Mazia (1936) and that sodium or potassium chloride solutions might act similarly is supported by instances in which calcium release is reported (Heilbrunn, 1937, chap. 33). Mazia (1937) has further shown by direct analysis that there is upon fertilization a decrease of about 15 % in the proportion of bound calcium in *Arbacia* eggs.

The failure of calcium chloride to activate eggs of *Nereis* is not in conflict with Heilbrunn's or Pasteels' views, since it may very well be due to failure or extreme slowness of penetration. Also the apparently paradoxical action of citrate which inhibits activation by other agents but results in activation when the eggs (*Nereis* or *Barnea*) are returned to sea water is explained by assuming that removal of calcium from the cortex makes it much more permeable to that ion.

Since the time of Just's (1919, 1923) and Chambers's (1921 *b*) finding that egg fragments devoid of cortex cannot be fertilized, there has been considerable discussion as to the significance of a cortical change in activation. Recently Moser (1939 *a* and *b*, 1940) by observation on centrifuged eggs revealed some new features of the cortical reaction and also correlated it with the presence of calcium. He describes in the cortex of the egg of *Arbacia* (see Fig. 6) a single evenly distributed layer of colourless granules that are not displaced by centrifugation and are clearly visible, particularly in the hyaline zone. Upon fertilization the cortical granules disappear within 10–20 sec., starting at the sperm entrance point, and a wave of crenation or slight roughening of the egg surface accompanies the granule breakdown. In cases where subsequent membrane elevation does not proceed smoothly, the points at which the membrane remains in contact with the surface can be correlated with the presence of intact granules. Treatment with artificial activating agents (saponin, toluol, puncture, ultra-violet, and direct current) likewise causes granule breakdown, but not if the eggs are given a preliminary treatment with oxalate or citrate. The cortical response to the artificial agents can occur in complete absence of oxygen (Kitching & Moser, 1940).

Moser considers the granule breakdown to be analogous to the surface precipitation reaction (Heilbrunn, 1928, 1937, 1940), in which the exudated contents of crushed eggs show a gelation or clotting accompanied by some granule breakdown producing vacuoles and the formation of a new limiting surface film. In the absence of calcium this reaction fails to occur. Elevation of the normal fertilization membrane is interpreted, then, as due to the cortical granules giving rise to vacuoles which enlarge and coalesce beneath the plasma membrane, so that the vacuolar film toward the periphery becomes the inner margin of the fertilization membrane, while the film on the side towards the egg becomes the new surface. Apart from the theoretical side these observations, along with a recently described pigment-release reaction (Churney & Moser, 1940), are of much importance in furnishing a clear picture of the hitherto poorly defined cortical change. It is of further interest in that they can be correlated with the calcium theories of activation.

The "sensitization to calcium" theory of Pasteels and the "calcium release" theory of Heilbrunn are primarily in accord in assigning a central role to calcium. The latter has the advantage of being somewhat better defined, fitting more of the facts, and covering general stimulation phenomena besides activation. It can also be related to Lillie's scheme (sect. VIII 5) for activation, and is in agreement with Hollaender's results (sect. VIII 4) indicating that a protein may be involved. But much remains to be done before anything like a clear picture may be had of the manner in which calcium may fit into the series of changes that are initiated upon activation of the egg.

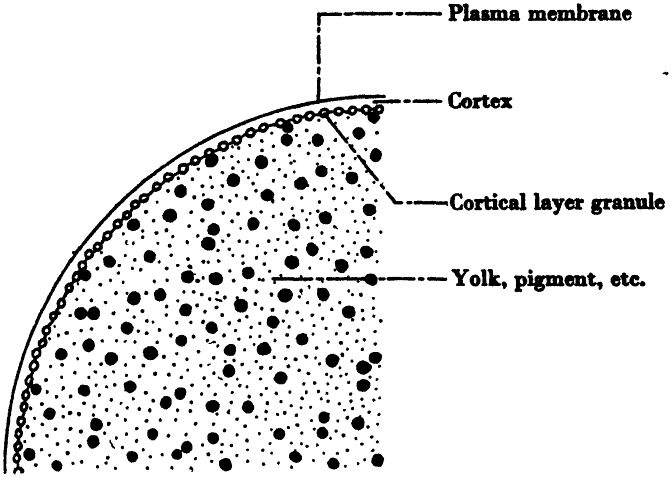

Fig. 6. Diagram of a quarter-section of an *Arbacia* egg. (From Moser, 1939*a*.)

7. *Physiological changes upon activation*

The changes that occur in various physical properties and in rate and kind of metabolism have been investigated primarily in connexion with activation by sperm (i.e. normal fertilization). While this work cannot be treated in the present review it must undoubtedly be taken into account in a really thorough analysis of the activation process. Reference may be made here to a few of the more recent papers containing information on changes in various properties of the egg upon fertilization. Studies have been made of the changes in rate and kind of metabolism (see Lindahl,

1938*b*, 1939; Brock, Druckrey & Herken, 1938; Laser & Rothschild, 1939; Örström & Lindberg, 1940; Ballentine, 1940; Needham, 1939; Mossman, 1940), in permeability (Lucké, Hartline & Ricca, 1939; Whitaker, 1939; Simon, 1939; Jacobs, 1939) in *p*H and *r*H (Spek, 1937; Ries, 1938), in volume (Tyler, 1932*a*), in egg protein solubility (Mirsky, 1936), in electrical properties (Cole & Cole, 1936; Dan, 1936; Rothschild, 1938), in surface forces (see Harvey, 1938; Chambers, 1938; Chambers & Kopac, 1937), in birefringence (see Schmidt, 1937; Schmitt, 1939), in hydrolytic enzymes (see Linderstrom-Lang, 1939), and in viscosity (Heilbrunn, 1937, 1940).

In the few instances where artificial activation has also been employed, the particular property shows exactly the same change as it does upon fertilization provided that the treatment has been optimal. This is not at all surprising, especially where normal development follows artificial activation. Of more interest, perhaps, are those cases in which some property of the egg changes without incurring activation. Thus, for example, the rate of respiration of unfertilized eggs may be raised to or beyond that of the fertilized egg by means of agents such as oxidation-reduction dyes and substituted phenols (see Runnström, 1935*a*; Clowes & Krahl, 1936; Barron, 1939), but the eggs are not activated by the treatment. One might then conclude that the respiration is not primarily involved in the activation process, and this accords with the fact that fertilization or artificial activation can occur under anaerobic conditions (see Kitching & Moser, 1940). The change in respiratory rate that normally occurs upon activation is evidently dependent upon other changes in the egg. Runnström (1937) has developed the view that the rise that occurs in the sea-urchin egg upon activation is due to a more intimate contact between catalysts and substrate or to the liberation of a mediator.

In two recent experiments (Brachet, 1938; Tyler & Horowitz, 1938*a*) advantage was taken of the fact that eggs may fail to divide even after optimal activating stimulus (sect. V9), in order to study the rise in respiratory rate that occurs during early development. These eggs (*Chaetopterus* and *Urechis*) showed a greatly diminished rise, which could be correlated with the retarded increase in nuclear material. Thus while the respiration quite likely supplies the energy for the developmental processes, it, in turn, is determined by other changes that occur during development.

IX. SUMMARY

1. The work on artificial parthenogenesis appearing principally in the past decade is reviewed in this article.

2. In a number of different species of animals artificially activated eggs have been reared to an advanced stage and even to sexual maturity. Once normal embryos are produced only the ordinary culture difficulties stand in the way of obtaining the adults.

3. The frequency of normal development, even after optimum stimulation, is quite low. This can often be attributed to such factors as haploidy, irregular chromosome distribution and lack of a proper division mechanism. In certain

instances, where other factors are apparently normal, it appears to be due to lack of a bilateral symmetry inducing action normally provided by the sperm.

4. In artificially activated eggs of certain forms (amphibia, echinoids) the cleavage amphiaster is fairly certainly derived from a cytaster. In the frog's egg the presence of foreign nuclear material is reported to be necessary for the formation of an amphiaster-producing cytaster. In the sea-urchin egg, cytasters capable of division can be produced in fragments devoid of a nucleus.

5. Non-nucleated egg fragments have been shown to be capable of undergoing repeated cleavages. This work is discussed in relation to central bodies and cytasters and the possible *de novo* formation of self-perpetuating bodies in general.

6. In marine animals other than the echinoids, cytasters have rarely been described. In several of these the amphiaster has been shown to arise as a result of suppressed polar-body divisions and probably involves renewed ability of the egg and polar-body central bodies to divide. The evidence shows a general incapacity for division on the part of artificially activated eggs which have extruded both polar bodies. Suppression of polar divisions leads to cleavage by (1) conversion of the presumptive polar spindle into a cleavage spindle, or (2) submerged polar divisions and subsequent fusion of nuclei and association of central bodies. The latter method can give rise to an amphiaster, triaster or tetraster. The type of behaviour is correlated with exposure time and activating agent.

7. Parthenogenetically produced individuals that have reached late or adult stages of development have the diploid, or sometimes higher, chromosome number. Regulation to diploidy has been shown to occur in a number of ways. In the frog and the silkworm doubling may occur at the first or a later cleavage, probably by fusion of daughter nuclei; occasionally perhaps by polar-body suppression. One investigation on the sex of parthenogenetic silkworms points to fusion of egg nucleus and a polar-body nucleus or omission of the reduction division as the general method of regulation. In *Urechis* utilization of the polar spindle for cleavage results in diploidy.

8. The sex of parthenogenetic frogs, silkworms and rabbits is shown to accord in the main with the expectations based on whether the species is male or female digametic and on the method of regulation to diploidy.

9. Activation has not as yet been accomplished by sperm extracts, but other specific actions on eggs have been demonstrated. There has been ample confirmation of the older gynogenesis experiments, showing the retention of activating capacity by the sperm after destruction of its genic material.

10. A new method of activation consists in the use of monochromatic ultraviolet light, and the results indicate that a protein may be involved in the process.

11. A "bimolecular" theory of activation has been developed by R. S. Lillie on the basis of differences in the conditions of action of heat and acid on the one hand and hypertony on the other.

12. Experiments with unbalanced isotonic salt solutions on normal eggs and with a variety of activating agents on "calcium-free" eggs have brought out the importance of calcium and are interpreted on the basis of two rather concordant views: Pasteel's "sensitization" theory and Heilbrunn's "calcium release" theory.

13. The same initial changes in metabolism and other properties occur upon artificial activation as upon fertilization. In spécial cases, such as when cleavage fails even after adequate initial stimulus, the later metabolic changes are correspondingly altered. Thus the normal rise in respiratory rate during development is shown to be dependent on changes accompanying cleavage.

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XI. ADDENDUM

A monograph by Astaurov (1940, "Artificial parthenogenesis in the silkworm *Bombyx mori*, L." Publications of the Academy of Sciences, U.S.S.R.) and two articles by Frolowa (1940, "Peculiarities of maturation of unfertilized eggs of *Bombyx mori*, L. activated by high temperature," *C.R. Acad. Sci. U.R.S.S.*, **27**, 601, and "Cytology of development of parthenogenetic eggs of *Bombyx mori*, L. activated by high temperature," *C.R. Acad. Sci. U.R.S.S.*, **27**, 604) were received after this review had gone to press. Frolowa presents cytological evidence supporting Astaurov's view that the reduction division is eliminated in the heat activated eggs.

Further work on the role of calcium is reported by Wilbur (1941, "The stimulating action of citrates and oxalates on the Nereis egg," *Physiol. Zoöl.* **14**, 84).

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